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Organic acid production by Aspergillus niger

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Organic acid production by *Aspergillus niger*

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Acknowledgements

Summary

The environmental impetus to move away from traditional chemical techniques towards biologically based production has revealed *Aspergilli* as very attractive cell factories. *Aspergillus niger* is particularly interesting for the production of organic acids, as it tolerates low pH, may ensure high conversion rates of the substrate to the metabolite of interest, can give high conversion yields and is already used extensively for production of organic acids (e.g. production of citric acid). All these factors should make *Aspergillus niger* an ideal candidate for metabolic engineering. However, the application of metabolic engineering for production of high concentrations of different organic acids has been limited by the lack of detailed knowledge on the central carbon metabolism and its regulation. The aim of this Ph.D. was therefore to develop and apply the necessary metabolic engineering tools for the study, and eventual manipulation, of the central carbon metabolism towards enhanced organic acid production. Several techniques related to enhanced genomic manipulation, intracellular metabolite profiling and fermentation were either developed specifically in this work, or transferred from work on other organisms. The application of metabolic engineering to *A. niger* allowed for the development of several strains with enhanced citrate production capabilities. The mechanisms underlying the increased productivities were also carefully investigated and described in detail. Finally, a proof-of-concept application of a novel reversible auxotrophic marker system was demonstrated through the deletion of a global acetate regulatory gene (*acuB*). The effects of this deletion on organic acid production are also discussed.

Dansk Sammenfatning

I den kemiske industri er der stor interesse for at udvikle bioteknologiske processer til production af kemikalier, idet disse processer er mere miljøvenlige og er baseret på anvendelse af fornybare resourcer. I denne forbindelse har *Aspergilli* vist sig at være attraktive som cellefabrikker. Specielt *Aspergillus niger* er interessant i forbindelse med produktion af organiske syrer, idet denne organisme tolerer lavt pH, kan give høje produktudbytter, og kan give høje produktiviteter som allerede illustreret i anvendelsen af denne organisme i produktionen af citronsyre. Disse faktorer gør *A. niger* til en ideel kandidat for metabolic engineering, men anvendelsen af metabolic engineering til at udvikle en *A. niger* cellefabrik der producerer forskellige organiske syrer har været begrænset af vores kendskab til metabolismen og dens regulering i denne organisme. Formålet med dette Ph.D. stadium har derfor været at udvikle og anvende metabolic engineering værktøjer til at foretage genetiske ændringer så den central kulstofmetabolisme understøtter produktion af organiske syrer. Således er der blevet udviklet og anvendt metoder der muliggør forbedret genomisk manipulation, måling af intracellulære metabolitter samt kontinuert fermentering af *A. niger*. Ved anvendelse af metabolic engieering lykkedes det at udvikle nogle stammer af *A. niger* der havde forbedret produktion af citrat. Mekanismerne bag de forbedrede produktiviteter blev undersøgt og resultaterne heraf er diskuteret i afhandlingen. Endelig blev et proof-of-concept anvendelse af en ny teknik til deletion af flere gener i genomet demonstreret ved at deletere det globalt acetate regulatoriske gen *acuB*.

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Introduction

The environmental impetus to move away from traditional chemical techniques towards biologically based production has revealed *Aspergilli* as very attractive cell factories. *Aspergillus niger* is particularly interesting as a cell factory for the production of organic acids, as it tolerates low pH, may ensure high conversion rates of the substrate to the metabolite of interest, can give high conversion yields and is already used extensively (e.g. citric acid). All these factors should make *Aspergillus niger* an ideal candidate for metabolic engineering. However, a limiting factor in applying metabolic engineering for production of high concentrations of different organic acids has been the lack of detailed knowledge on the central carbon metabolism and its regulation. That is also why there has been very limited success in the metabolic engineering of *A. niger* towards increased production of organic acids, with one of the few examples being the increase in citrate productivity by disruption of Trehalose-6-phosphate synthase A[i].

The aim of this work was therefore to develop and apply the necessary metabolic engineering tools for the study, and eventual manipulation, of the central carbon metabolism towards enhanced organic acid production. The general course of this investigation can be seen in figure 1.1.
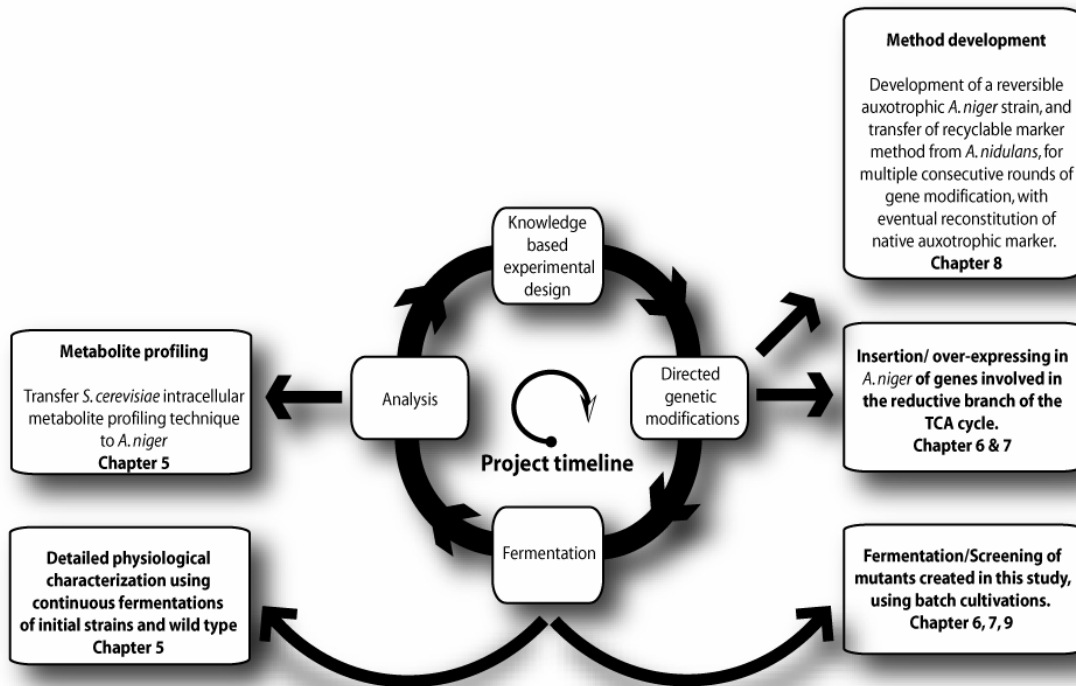
Figure 1.1 Timeline of this Ph.D.: Starting with the physiological characterization of strains through continuous fermentation and continuing to the final characterization of all mutants created during this Ph.D. Some details are also given regarding the different projects and method development activities, which formed part of the overall work.

The dissertation starts with three chapters (chapters 2-4) devoted to introducing the various fields of interest for this work. Chapter 2 is a review of current literature with its main focus on efforts towards enhancing citrate production in the past, present and near future. This chapter is intended to present examples of the importance and general applicability of studying organic acid production in *A. niger*. It is presented as a shortened version of the book chapter "*Filamentous fungi as cell factories for metabolite production*, which was written during this Ph.D. Chapter 3 serves as an introduction to the currently available molecular biology techniques for *A. niger*, as well as the limitations of these techniques, as they were encountered at the start of this Ph.D. The final introductory chapter, chapter 4, explains the advantages, as well as practical difficulties, of using continuous fermentation to study the physiology of filamentous fungi.

The introductory chapters are followed by 3 chapters (chapters 5-7) presenting the results obtained in the process of studying organic acid production in *A. niger* through various means. Continuous fermentations, along with intracellular metabolite profiling, were employed, and the results are presented in chapter 5. This chapter is written in the format of a paper and will be submitted to an international journal. Chapter 6 is devoted to laying out the methodology, and results from several experiments, which were employed in an effort to obtain a succinate overproducing strain of *A. niger*. This chapter was written in a book chapter format and does therefore not contain much detail on the materials and methods used. The details of strain construction and general batch fermentation techniques employed, however, can be found in chapter 7. Chapter 7, which is written in the format of a paper, presents the study, and successful increase in productivity, of citrate production in *A. niger* through several deferent gene-insertions. Both chapters 6 and 7 are included in a patent application based on this work.

Chapter 8 presents the proof-of-principle of a novel method developed to enable multiple consecutive genomic manipulations to be performed, with eventual reconstitution of the native auxotrophic gene, in *A. niger*. This technique also includes the first application of the PCR based, cloning free, bi-partite technique, which both increases the number of correct transformants obtained and reduces the time involved in creating a new mutant. This chapter was written as a paper and will be submitted to an international journal. The final results chapter shortly summarises some interesting results obtained for the ΔacuB strain (the first created through deletion, apposed to mutation, in *A. niger*) created as part of the proof-of-principle work in chapter 8.

Finally, chapter 10 serves as a summary of all the results presented in this dissertation.

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[i] Arisan-Atac I, Wolschek MF, Kubicek CP. 1996. Trehalose-6-phosphate synthase A affects citrate accumulation by *Aspergillus niger* under conditions of high glycolytic flux. FEMS Microbiol Lett 140(1):77-83

Chapter 2: Filamentous fungi as cell factories for metabolite production

2.1 Introduction

Filamentous fungi are extensively used as cell factories for different biotechnological products such as enzymes, chemicals and food ingredients. The two most eminent biotechnological processes are citrate production by *Aspergillus niger* and penicillin production by *P. chrysogenum*. This chapter will focus on citrate production, which in terms of industrial production represent billions of US$ in annual revenues.

Hyperproduction can be defined as the ability of an organism to obtain very high yields, in some cases close to stoichiometric conversion of a carbon source into the product of interest in a relatively short period of time (generally a few days). Such intensive production can only be obtained with a few selected isolates of filamentous fungal species under specific nutrient and morphological conditions.

The original natural isolates would be considered poor producers by modern standards. However, intensive efforts have been applied to their improvement, using mostly classical random mutation strain improvement techniques, and more recently genetic and metabolic engineering techniques. Concurrent to the advances in fungal genome manipulation techniques, great strides forward in the development of comprehensive metabolite detection, large scale mRNA transcript quantification and other so called "omic" techniques have been made. The application of these techniques to strain improvement is already starting to revolutionize the way in which we improve and develop current or novel processes. This chapter will discuss these developments after a review of the historical development of citrate production, an example where hyperproducing filamentous fungi have been developed.

2.2 History

From a historic perspective, citrate production by *A. niger,* fumarate production by *Rhizopus oryzae*, and penicillin production by *Penicillium chrysogenum*, have great importance as the first true examples of industrial scale biotechnological processes. These fungal fermentation processes have led the way towards further industrial applications of biotechnology. Particularly the development of citrate and penicillin production processes resulted in mutual advantages, and led to rapid progress in the field of biotechnology as a whole. In the course of developing these processes many similar problems arose, and hence many of the techniques developed for one process could rapidly be transferred to the other process (see figure 2. 1 for a timeline overview of key historical developments), e.g. large-scale fermentation technology.

Citrate was first discovered by Scheele in 1784 and was produced mainly from lemons during the 19th century. In 1880 it was attempted to produce citrate from glycerol, but this was not economically competitive. As early as 1893 there was the first hint at using a biotechnological process when Wehmer discovered a mold that produced citrate (Mattey, 1992). He called it *Citromyces*, which later became better known as *Penicillium*. In 1917 Curie found an *A. niger* capable of producing high concentrations of citrate in a sugar medium. This discovery quickly led the US company, Chas. Pfizer & Co. Inc., to start producing citrate on a large scale using surface cultures. The process was soon improved by using beet molasses instead of sugar, thereby making the process more economically viable.
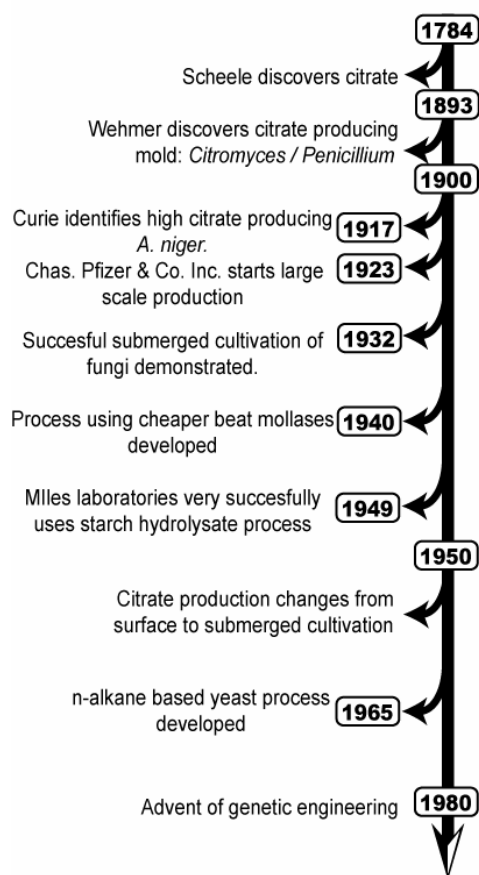
Figure 2. 1: Timeline of key historical developments for citrate production.

In connection with design of large-scale penicillin production there were several problems to be solved to achieve successful submerged cultivation. These included: sterile design, dirt and oil free equipment design, large scale agitators and sterile aeration capabilities (Shuler, M. L. and Kargi, 1992). Similar problems were encountered for citrate and penicillin production, but in the case of citrate production sterile air was less of a problem, as the very low pH at which citrate fermentations are conducted makes contamination much less likely than for penicillin fermentations. Successful submerged penicillin production was rapidly achieved, which is generally attributed to the transfer of know-how from the submerged fumarate production process using *R. oryzae*. The *R. oryzae* process was the first submerged fermentation process using filamentous fungi and was therefore used as a model for scale-up and fermentation techniques for the penicillin production process (Roehr, M. and Kubicek, 1996). Hurdles in submerged cultivation were overcome by a multidisciplinary team, which included biologists and chemical engineers, and can be seen as the birth of biochemical engineering as a discipline.

These advances in fermentation technology soon started to be applied to the production of citrate, and from 1950 the process changed over time from surface to submerged cultures. In 1965 various yeast based citrate production processes were introduced. These processes used n-alkanes, since hydrocarbons were cheap at the time, but most have now changed to regular carbon sources (Barbesgaard *et al.*, 1992). Furthermore, today *A. niger* is the main cell factory used for citrate production.

Rapid progress was made in the optimisation of fermentation parameters during the following years leading to large increases in yields and productivities for both citrate and penicillin. Process improvements using classical strain improvement approaches to obtain higher producing strains soon started and this was the main approach to this aim up to the 1980's. DNA-mediated transformation started in the mid 1980's and quickly led to new disciplines such as genetic engineering, and later, metabolic engineering.

Citrate and penicillin production techniques are still being improved today, and it is a measure of the historical, as well as current importance of these products, that they

still generate such interest. Figure 2. 2 shows the increase in the world consumption of citrate. Table 2.1 provides some additional economic data on citrate production.

The fast increase in citrate consumption along with its current and expanding global production capacity has placed citrate as one of the largest bulk commodity products produced by biotechnology worldwide.

Table 2.1: Citrate production statistics

	Citrate *A.niger'*
World market[million $] "	360
Cost [US$/kg]	0,4
Titer [g/L]	>110'''
Yield [%]	>80'''

' Comparatively small percentage of citrate produced by other organisms.

" For the year 2000

''' Mattey, 1992
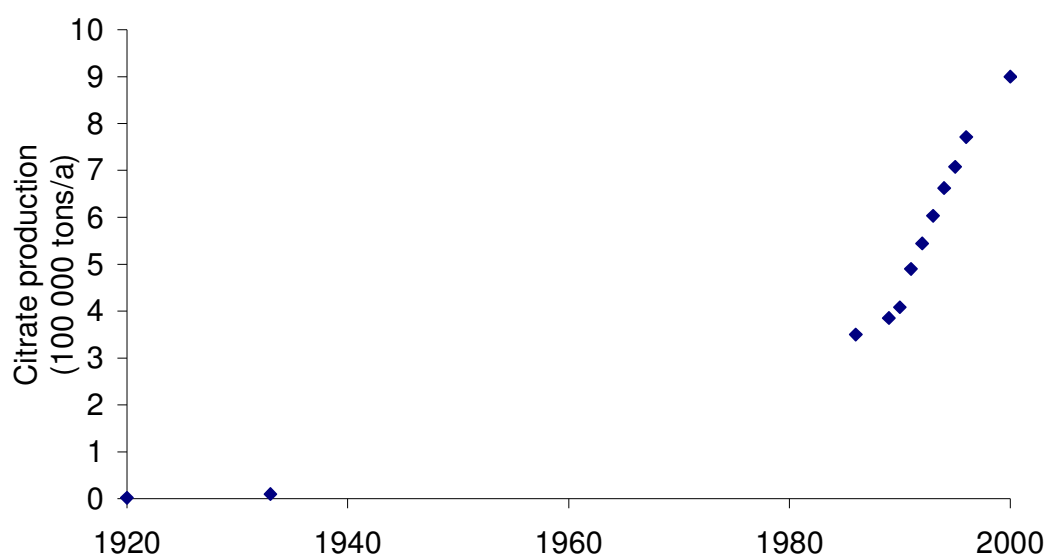
Figure 2. 2: World market development of citrate.

2.3 Producer strains

Several producer strains are currently employed in industrial processes: citric acid and gluconic acid production by *A. niger*, penicillin by *P. chrysogenum*, itaconic acid production by *A. terreus*, malate and fumarate production by *Rhizopus oryzae*, as well as numerous enzyme production processes. Table 2.2 gives a list of products of interest and the organisms that produce them. An example of a filamentous fungi, in the form of *Aspergillus niger* sporulating on agar plates, are presented in figure 2.3.

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Table 2.2. Products of interest and the fungi that produce them (Adapted from (Ruijter, G. J. G., Kubicek, and Visser, 2002), with additional information from (Roehr, M., Kubicek, and Kominek, 1992) and (Mattey, 1992))

Organism	Metabolite	Application
A. niger	Citrate	**Food &beverage industries** Acidifier, pH adjustment, flavour enhancer, reduces sweetnes, antioxidant, preservative **Pharmaceutical industry and Cosmetics** pH adjustment, Anticoagulant, Antioxidant, fast dissolution of active agent, preservative in stored blood, iron citrate as a source of iron **Others** Cleaning of metal surfaces, oil well treatment, retards concrete setting, hardening of adhesives, Plastics industry, washing agents, household cleaners, removal of sulfur dioxide from waste gases, leather tanning, electroplating
	Gluconate	Food additive, therapeutic metal salts, dissolving of calcium deposits such as milkstone in dairy industry, metal cleaning
A. terreus	**Itaconic acid**	**Plastics and paper industries**
R. oryzae	**Fumarate**	**Food additive, Synthetic polymers**
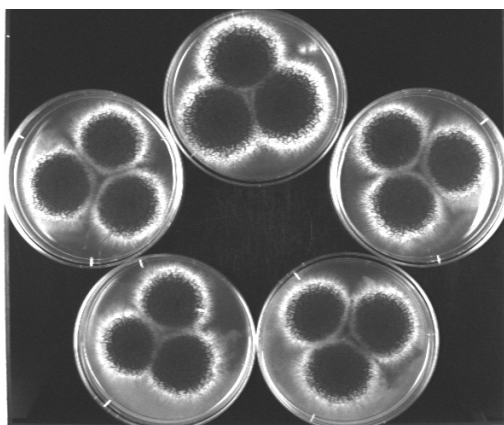
Figure 2.3. *Aspergillus niger* **sporulating on agar plates.**

2.3.1 Platform organisms

Even though there are several fungal producer strains used at the moment by industry for organic acid and antibiotic production, only *A. niger* and *P. chrysogenum* can be characterized as platform organisms for metabolite production. A platform organism is an industrial organism that is extensively used to produce several commodity products. The main advantages of these organisms are that they are well understood and have been used by industry for an extended period of time. This allows rapid transfer of new products from the research and development stage to production. Table 2.3 lists the advantages and disadvantages of three industrially used producer strains.

Table 2.3: The advantages and disadvantages of producer strains used by industry.

Organism	Advantages	Disadvantages
A. niger	High acid tolerance (>200 g/L citrate) High productivities possible (r_p> 1.4 g/L/h) Can utilize complex substrates (biomass) Extensively applied in industry GRAS organism 4 x coverage of genome publicly available, public sequencing project to 9 x coverage will start in 2005.	Does not naturally produce other useful organic acids (other than citrate and gluconate) at high titers Can be consumer problems with GM strains.
A. terreus	Used to produce Itaconic acids at high titers and productivities (15,000tons/a) Itaconic process is considered GRAS In process of being sequenced for a public assessable database	Less industrially utilized than *A. niger* Seen as an emerging antigen for causing aspergillosis, and resistant to amphotericin B, a crucial treatment for fungal infections (Steinbach *et al.*, 2004).
P. chrysogenum	High titers of β-lactams possible (>50 g/L) High productivities Hyperproducers can be used as platforms for new products (ad-7-ADCA) Extensively applied in industry GRAS organism	Not yet sequenced Only used for secondary metabolite production

2.3.2 Health and safety

Both *A. niger* and *P. chrysogenum* are particularly suitable for food or drug production, as they have been certified as generally regarded as safe (GRAS) by the Food and Drug Administration (USA) and the World Health Organisation. Many specific *A. niger* production processes have been certified as GRAS, among these are several enzymes: α-amylase, cellulase, amyloglucosidase, catalase, glucose oxidase, lipase and pectinase. Bulk chemicals produced by *A. niger* have also attained this status, such as citrate and gluconate. In the case of *P. chrysogenum,* production of penicillin and adipoyl-7ADCA has also been recognised as GRAS.

As is the case for other fungi, it is important to avoid too much contact with the spore dust, but *A. niger* (Schuster *et al.*, 2002) and *P. chrysogenum* are not considered particularly dangerous. *A. niger* has only been found to cause human infection in people with a compromised immune system, and while Ochratoxin A is produced by *A. niger* in rare cases (Schuster *et al.*, 2002), it has not been cited as a problem for any of the current biotechnological applications.

The safety and ease of use of these platform organisms has led to their extensive application in industry and has made them the focus of intense study and process improvement projects.

2.4 Classical strain improvement

Classical strain improvement has been used since the early 1950's to increase yield, titer, productivity or general ease of use of *Aspergillus niger* and *Penicillium chrysogenum*, and has been responsible for virtually all the published hyperproducers to date. Mutagens such as short wavelength radiation (UV), X- and γ-ray ionizing radiation and chemical agents (base analogs: 5-chlorouracil, hydroxylamine; alkylating agents: N-methyl-N'-nitro N-nitrosoguanidine; intercalating agents ect.) have been extensively used. The most popular agent of these, because of its very high mutant to survivor ratio and multiplicity of mutations, has been nitrosoguanidine (Parekh *et al.*, 2000). Most mutations occur at very low frequency (10^{-5}-10^{-10}/ generation, Parekh *et al.*, 2000) and mostly with negative impact on the characteristic of interest. The challenge is to isolate the true beneficial mutants, and therefore, well-designed screening and directed mutagenesis techniques are of vital importance.

Random mutation and selection were initially used because the lack of any detailed biochemical knowledge of the organisms prevented a more directed approach. This empirical method can be described as "hit and miss", requiring a brute force approach. A classical case would involve the screening of more than 100,000 mutants to obtain a positive result (Lein., 1986).

By applying **rational selection techniques** a 10 fold improvement in hit rate can be achieved (Vournakis and Elander, 1983), which results in a direct efficiency increase for strain improvement programs. Rational selection techniques involve screening, not for the product of interest, but for a biochemical characteristic associated with it. Such techniques involve, for example, direct colony selection after a bioassay overlay for better penicillin producers (Vournakis and Elander, 1983), or citrate specific

indicator (P-di-methylaminobenzaldehyde) assays for citrate hyperproducers (Mattey, 1992).

2.4.1 Resistance to metals

Metallic ions are of great importance to citrate production. It has long been known that the presence of even trace amounts of Mn^{2+} (more than 1 ppb, Mattey, 1992) will drastically decrease citrate production in submerged cultivation. Mn^{2+} has been reported to have multiple effects on *A. niger* physiology, *e.g.* morphology changes from pellet to filamentous, increased protein turnover, impaired DNA synthesis and altered composition of plasma membrane and cell walls (Karaffa and Kubicek, 2003). Traditionally, the feedstock has been de-ionised using ion-exchange pre-treatment, which adds to the labour and costs involved in using substrates such as molasses. Classical selection techniques have therefore been used to select for mutants less sensitive to Mn^{2+} [(Lesniak W. *et al.*, 2002); (Schreferl Gerlinde *et al.*, 1986); (Gupta Sanjay and Chandra B.Sharma, 2002)].

2.4.2 Decreased by-product formation

Selecting mutants deficient in the responsible enzymes can be used to decrease unwanted by-product formation. This has the added advantage of increasing process productivity, while hopefully increasing yields. Unwanted gluconate and oxalate productions were prevented in this way by selecting for mutants deficient in glucose oxidase or oxaloacetate hydrolase, respectively (Ruijter *et al.*, 1999). A double mutant obtained by Ruijter *et al* (1999) also had the unexpected characteristic of being able to produce citrate at high pH, and in the presence Mn^{2+}.

2.5 Metabolic engineering

> *"The combination of analytical methods to quantify fluxes and their control with molecular biological techniques to implement suggested genetic modifications is the essence of metabolic engineering."* Stephanopoulos, G. N. Aristidou A. A. Nielsen J., (1998)

Metabolic engineering can be divided into two main branches: Improvement of current processes and new product development (See figure 2. 4). These can be seen as the next logical biotechnological step following on classical strain improvement and genetic engineering. Genetic engineering, which can be defined as the transformation of a cell by foreign DNA, supplied the necessary tools for the directed genetic changes, which is a central theme of metabolic engineering (figure 2. 5).
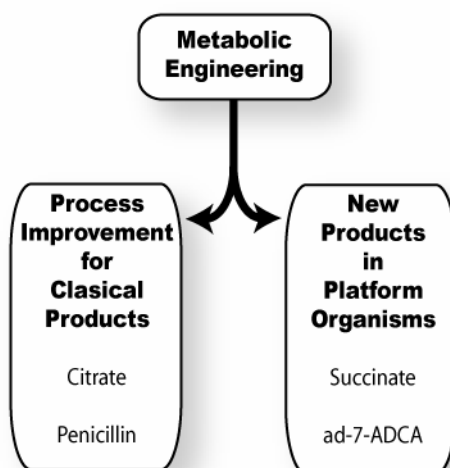
Figure 2. 4. The two main branches of metabolic engineering.
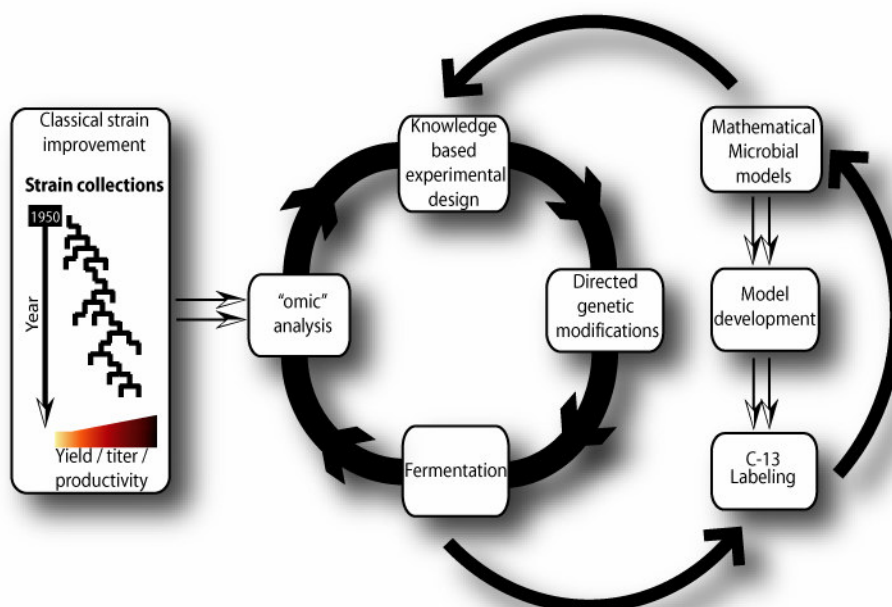
Figure 2. 5. Process improvement through metabolic engineering is an iterative approach, where, using all available information, several rounds of directed strain improvements, and in-depth experimental analysis, leads to a strain with preconceived physiological attributes. The importance of classically improved strain lineages becomes apparent when this approach is applied to citrate production. The information obtained from the in-depth study of these strain lineages can be directly applied to better design strategies for directed strain improvement.

2.5.1 Towards a systems approach

Unfortunately, researchers on citrate production have not always been very successful in translating knowledge gained from classically improved strains through metabolic engineering. In most cases these studies demonstrate one of the fundamental difficulties in metabolic engineering, namely the need for extensive and detailed information about the process, pathway or metabolic branch point one wishes to manipulate. Promper *et al.* (1993) investigated the role of complex I (NADH:ubiquinone oxidoreductase) in citrate accumulation. The study was

undertaken following the observation that the mutant citrate- producing B60 strain lost complex I at the onset of citrate accumulation (Wallrath *et al.*, 1991). The NADH binding subunit of complex I was therefore deleted in strain nuo51, which then disrupts the assembly of a functional complex I, and compared to the strain B60 and the parent. As expected the intracellular citrate concentration increase over that of the parent strain (up to 20 fold), but unexpectedly the total citrate produced was much reduced. The reason for this could not be proven, although the authors speculated that it was probably the lack of a citrate transporter, possibly mitochondrial, which led to strain nuo51 growing much slower than either the parent or B60, and that this lack also accounted for the low citrate production.

Another example was the improvement of the flux towards citrate production by over-expressing pyruvate kinase and phophofructokinase (Ruijter *et al.*, 1997b). The rational for this approach was mainly based on previous work, which suggested that hexokinase and phosphofructokinase may be important steps in flux control (Schreferl-Kunar *et al.*, 1989). It has also been found that high-producing strains, selected for their ability to grow faster in high sucrose concentrations with increased citrate yields, had two fold higher activities of these two enzymes (Schreferl-Kunar *et al.*, 1989). These conditions of high sucrose concentration have also been found to increase intracellular fructose 2,6-bisphosphate concentration (Kubicek-Pranz *et al.*, 1990), which is in turn a potent activator of phosphofructokinase. Although the approach was well thought out, and the necessary tools were available, an unexpected compensatory mechanism negated the genetic modifications. Over-expression of citrate synthase also had no effect on citrate production, most likely because the native citrate synthase had excess capacity (Ruijter *et al.*, 2000a).

2.5.2 Modelling in metabolic engineering
The above mentioned study on improving citrate production through over-expression of key genes in the pathway clearly points to the fact that the use of directed genetic changes can be problematic without systems knowledge. A major step towards applying a systems approach in metabolic engineering is arising in the form of using genome-scale microbial models (Patil *et al.*, 2004b). Two major classes of models are of current interest: Kinetic models and Stoichiometric models.

Both of these modelling approaches offer directly applicable advantages for metabolic engineers. Kinetic models represent a dynamic view of the cellular metabolism, but its application is severely constrained. The main reason for this is the lack of enzymatic kinetic data, which are cumbersome to obtain, and the discrepancy between *in vivo* and *in vitro* enzyme activities. These problems have limited the use of kinetic models for large metabolic networks, but, interesting results have been obtained after extensive study of specific and relevant pathways, such as those for citrate [(Guebel D.V. and Torres Darias N.V., 2001); (Torres N.V. *et al.*, 1996). Varez-Vasquez *et al.* (2000) predicted a 5 to 10 fold increase in citrate production rate through the over-expression of 13 genes or more. This is currently outside the possible practical range of genetic modifications and a reworking of this problem, using a macroscopic approach and different physiological parameters (Guebel D.V. and Torres Darias N.V., 2001) suggested a less extensive manipulation of the strain. It was predicted that with over-expression of the glucose carrier alone, a significantly increased citrate production rate could be achieved. Unfortunately, Papagianni and Mattey, (2004) have demonstrated that glucose import during citrate production is a passive transport

process, and therefore over expression of the glucose carrier is likely to have little effect, unless a heterologous active transporter is expressed. Torres N.V. *et al.*, (1996) used biological systems theory coupled with constrained linear optimization, to show that at least 7 glycolytic enzymes needed to be over-expressed to achieve a significant increase in flux towards citrate. This could help explain why the single and double over expressions of glycolytic genes [(Ruijter *et al.*, 2000b); (Ruijter *et al.*, 1997a)] tried so far has had no (or little) effect.

Stoichiometric models describe the biochemical reactions in the cell as a set of algebraic equations, and can be used to simulate steady-state intracellular fluxes [(Henriksen *et al.*, 1996); (Patil *et al.*, 2004a)] (see Figure 2. 6). The main advantages of stoichiometric models compared with kinetic models are: No kinetic information is needed, comprehensive models of cellular biochemistry are possible, and these models can be used to test pathway insertions or deletions, *in silico*, with relatively little effort [(David *et al.*, 2003),(Forster *et al.*, 2003)]. This last feature of stoichiometric models is particularly useful for metabolic engineering, as it allows a systems approach for planning directed genetic changes. A recent stoichiometric model of the central carbon metabolism by David *et al.*, (2003) is the most comprehensive effort to date for *A. niger* and includes more than 230 reactions. The power of large scale stoichiometric models have been illustrated through the work of Forster [(Forster *et al.*, 2002); (Forster *et al.*, 2003)] in *S. cerevisiae* , where it was shown to be possible to predict intracellular fluxes with high accuracy for anaerobic conditions, and predict extreme pathways and maximum theoretical yields for aerobic conditions.
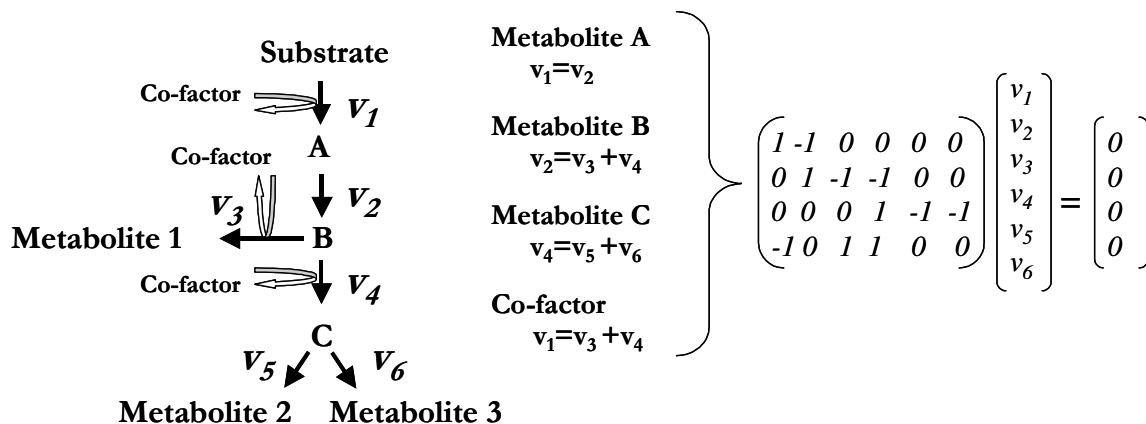
Figure 2. 6. Example of the mathematical formula representing the stoichiometric model of a simple branched pathway.

2.5.3 Carbon-13 labeling
Combining stoichiometric models with ^{13}C-labeling enables quantitative knowledge of the intracellular fluxes [(Christensen *et al.*, 2001); (Christensen and Nielsen, 2000a); (Christensen and Nielsen, 1999)], and can also lead to the elucidation of previously unknown pathways (Christensen and Nielsen, 2000b). This tool plays an important role in elucidating the major physiological differences between high and low citrate producers. It has also extended the understanding of the effects of glucose concentration on glycolytic metabolism under citrate producing conditions (Peksel *et al.*, 2002).

2.5.4 Examples of strain improvement through metabolic engineering

The high level of secrecy surrounding industrial producer strains means that we can only focus on the work done by academia. To date, there have been only a few success stories for the application of metabolic engineering to citrate production in *A. niger*. The public resistance to GM products probably has a dampening effect on the application of metabolic engineering for citrate production.

The only direct improvement in citrate productivity was achieved through the increased rate of citrate accumulation in the early stages of fermentation on sucrose. Knowing that *A. niger* hexokinase is inhibited by trehalose-6-phosphate *in vitro* (Panneman *et al.* (1998) later showed that it was a particularly strong inhibition) and reports that hexokinase is a flux controlling step (Panneman *et al.*, 1998), Arisan-Atac *et al.*, (1996) decided to study the *in vivo* relevance of this inhibition. Several *A. niger* strains were constructed, containing either an amplification or disruption of trehalose-6-phosphate synthase A (T6PSA)-encoding gene (*ggsA*). It was found that, although equal final concentrations were achieved, the disruptant strain accumulated citrate faster than the wild type strain, while the amplified strain led to slower accumulation. It therefore appears that decreased trehalose-6-phosphate leads to higher hexokinase activity, bearing out the *in vitro* results *in vivo*.

Decreased by-product formation is an important target for yield, titer and productivity improvements through metabolic engineering. Unlike the classical strain improvement approach, where mutation was employed as an imprecise tool for by-product reduction, metabolic engineering techniques allow for precision excision of offending genes and subsequent detailed analysis of the resulting strain. A good example of this is the cloning and deletion of the oxaloacetate hydrolyase (OAH) gene in *A. niger* by Pedersen [(Pedersen *et al.*, 2000c); (Pedersen *et al.*, 2000b)]. The deletion of OAH resulted in the elimination of the by-product, oxalate, which causes problems during the downstream purification of proteins. It was also shown through a carbon labelling study that such a deletion can be done without pleiotropic consequences (Pedersen *et al.*, 2000a).

The importance of morphology during citrate fermentations, and the negative influence of Mn^{2+} on the desired pelleted morphology, has been stressed earlier. Dai *et al.* (2004) used suppression subtractive hybridization to identify key genes involved in the negative effects of Mn^{2+} on pellet morphology in *A. niger*. Anti-sense expression of one of these genes (*Brsa-25*, a possible amino acid transporter) was then employed to allow pelleted growth in the presence of high Mn^{2+} concentrations.

Table 2.4. Examples of successful process enhancements through metabolic engineering

Metabolic Engineering aim	Strain	Strategy	Result
Productivity improvement			
Arisan-Atac *et al.* 1996	*A. niger*	Trehalose-6-phosphatase synthase A deletion	**Citrate** Increased in high conc. sucrose
Decreased by-product formation			
Pedersen *et al.* 1999	*A. niger*	Targeted deletion of oxaloacetate hydrolyase gene	**Oxalate** by-product eliminated
Lower sensitivity to trace Mn^{2+}			
Dai *et al.* 2004	*A. niger*	Target identification by suppression subtractive hybridisation, followed by antisense expression of *Brsa-25*	**Morphology** and **Citrate** Less sensitive to Mn^{2+}

Even though these successful improvements of hyperproducers have been achieved, the main limitation on advanced strain improvement remains a lack of fundamental insight into regulatory structures, optimally at the level of the complete system. As we have discussed earlier, mathematical models have moved the field closer to a systems approach, but the exclusion of regulation from metabolic flux models is a limitation for identification of metabolic engineering targets.

2.6 Post Genomic

The developments in genomics provide knowledge and techniques that can lead to better understanding of how cell factories operate. Beside genome sequencing, which by itself is potentially extremely valuable for metabolic engineering as the identification of genes and gene targeting is greatly facilitated, genomics encompass transcriptomics, proteomics, metabolomics, etc. Using the information gathered in these fields, and integrating it with mathematical models (Akesson *et al.*, 2004), it may generally lead to an improved systems approach to strain design.

2.6.1 Transcriptome
Aspergillus nidulans was the first filamentous fungus to have its genome sequenced. It was chosen because of its use as a model organism for genetics in filamentous fungi, and the long history of work in *A. nidulans* meant that all the tools necessary to take advantage of the genome sequence were readily available. The successful sequencing of *A. niger* was announced in a press release by DSM in 2001 and Affymetrix arrays have been created (see table 2.5). A 4 x coverage of the genome sequence, provided by Integrated Genomics (Genencor), has been publicly released in 2005, and a new public sequencing project, using the Integrated Genomics sequence as starting point, has been initiated in 2005 by the Department of Energy in the USA to improve the coverage to 9 x (http://www.jgi.doe.gov/sequencing/ DOEmicrobes2005.html). *A. terreus* is in the process of being sequenced (http://www.ebi.ac.uk/genomes/wgs.html) and *P. chrysogenum* is currently listed as a candidate for sequencing by the Fungal Genome Initiative, to date no set time table

has been put forward. At the moment about 2,500 ESTs (Expressed Sequence Tags) are publicly available for *A. niger* (Hofmann *et al.*, 2003), and a quick search on the NCBI website (http://www.ncbi.nlm.nih.gov/entrez/) gives in the region of 200 and 100 partially or fully sequenced and annotated genes for *A. niger* and *P. chrysogenum*, respectively.

Table 2.5: Genome characteristics for *Aspergillus niger* and *Aspergillus nidulans* (adapted from Archer *et al*, 2004)

	Aspergillus niger	*Aspergillus nidulans*
Genome size [Mb]	35.9	30.1
Predicted Genes	14097	9967
Genes with Pfam hits	5306	4512

Although no global transcription analysis publications are available for *A. niger* or *P. chrysogenum* or other industrially important fungi, the possibilities for strain improvement through this approach have already been amply demonstrated. For example: A limited sized array was used to analyse *A. oryzae* growth on solid media and in liquid culture; from this array it was possible to identify several genes involved in morphology (Hofmann *et al.*, 2003). Another possible result of great value would be the identification of global signal transduction pathways and global regulators, which have been achieved in such diverse organisms as *E. coli* [(Ma *et al.*, 2004a); (Ma *et al.*, 2004b)], *S. cerevisae* (Bro *et al.*, 2004) and *A. Nidulans* [(Brakhage, 1997b); (Litzka *et al.*, 1999b)].

2.6.2 Metabolome

> "It has become clear that even a complete understanding of the state of the genes, messages, and proteins in a living system does not reveal its phenotype. Therefore, researchers have started to study the metabolome (or the metabolic complement of functional genomics)." (Villas-Boas *et al.*, 2004)

In order to define the metabolic state of an organism increasing emphasis is being laid on the central role of mass spectrometry. An extensive review on this field and its application to phenotypical analysis is presented by Villas-Boas *et al.* (2004). Techniques, such as gas chromatography coupled with mass spectrometry, have been used to determine the phenotype of otherwise null-mutants in yeast, by identifying most of the relevant metabolites in the central carbon metabolism (Villas-Boas *et al.*, 2005). A study on the applications of metabolite profiling of fungi by Smedsgaard and Nielsen (2005) demonstrated how direct infusion MS and HPLC with diode array detection could be used to identify novel compounds. It was also shown to be possible to differentiate between closely related fungal species and gain a deeper understanding of the phenotypic behaviour of fungal and yeast strains. Detailed phenotypical analysis plays a central role in most modern strain improvement programs, and advances in the field of metabolite profiling is therefore of great interest.

2.7 Future perspectives

As more fungal species are sequenced, and our abilities in proteomics and metabolomics are perfected, it will become possible to extend the product range of our platform organism beyond what has been done so far, and also improve on existing industrial processes.

The citrate production process already has a yield of more than 80% (see table 2.1). Here the future aim is therefore predominantly to improve process productivity. Productivities of 0.7g/L/h-1g/L/h have been reported [(Mattey, 1992);(Roehr *et al.*, 1992)], and we presented an example where increased productivity was achieved for citrate production through the deletion of trehalose-6-phosphatase synthase A (Arisan-Atac *et al.*, 1996). Mathematical kinetic modelling predicts possible productivity increases in the range of 45% (if glucose transport into the cell can be doubled (Guebel and Darias, 2001) to over 500% (depending on the over-expression of 13 key enzymes and increases in enzyme concentrations (Torres *et al.*, 2000)). It is not currently practically possible to test these predictions by over-expressing so many enzymes in a balanced fashion, and many of the enzymes have not yet been identified, but in the future we can expect attempts to be made as technologies for controlled expression of genes improves.

Successful future strategies will also depend on the development of more complete mathematical models of the relevant hyperproducers, a process that should be greatly facilitated by the public release of the *A. niger* genomic sequence. It will also be important to improve the integration of data from several "omic" techniques with large-scale microbial models, as this should in future provide tools for a stronger systems approach to strain design.

The value of classical strain improvement methods will still remain, especially as some processes can currently not use genetically modified organisms, but it is clear that modern methods and techniques will play an ever-greater role in the development and improvement of current and novel processes in the future.

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Chapter 3: An Introduction to Gene-targeting

"In a general sense, genetic recombination methods include those techniques that produce organisms having stable, expressable, genetic traits obtained by combining genetic elements from two or more parental organisms. " (Vournakis J.N. and Elander R.P., 1983)

The manipulation of genomic DNA can be achieved through many different methods, two of which are of interest in this context, targeted integration and random integration. Targeted integration, or gene-targeting, as the name implies, mainly focuses on achieving specific changes to the genomic DNA with as few as possible pleiotropic effects. This is achieved through the use of native chromosomal DNA based, homologous targeting sequences, which, through homologous recombination, lead to the integration of the gene-targeting substrate into the correct locus (see figure 3.1 A). Random integration, on the other hand, focuses on introducing DNA into the genome without using native homologous targeting flanking sequences. This generally results in several randomly integrated copies of the heterologous gene into the chromosome of the transformed strain, through non-homologous end-joining (see figure 3.1 B).
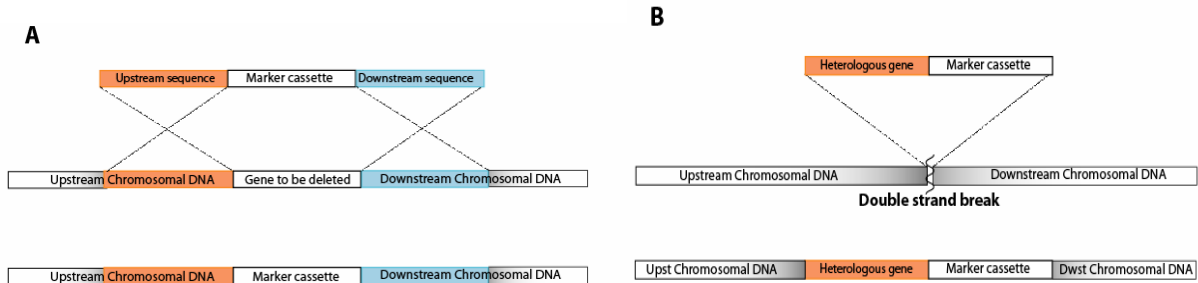
Figure 3.1 Two of the different modes of integration of foreign DNA into the genome: (A) homologous recombination and (B) random integration.

A specific danger of random insertion is the significant chance of a pleiotropic effect, such as disruption of a chromosomal gene, that has a central function and hereby the transformation negatively influences growth or production. This makes it essential to screen several transformants to ensure the observed effects are related to the gene insertion, and not a pleiotropic effect. The main reasons random integration are still employed are the ease of implementation of this strategy, as well as the often-important ability to attain high copy number insertions. High copy numbers, even when the gene is placed behind a strong promoter, can in some instances be the only way to observe its effect. It is also often the best strategy to obtain high productivities when a metabolic bottleneck is targeted.

There exists a method to obtain very high copy numbers encompassing the co-transformation of a marker containing plasmid and the heterologous gene bearing

plasmid (usually at a 1: 10 ratio). Such a co-transformation results in variable copy numbers in the transformants, and allows the study of the effects of increasing copy number on strain physiology. Several other strategies are available and can be seen in table 3.1, and while each strategy has its particular purpose, there are also associated advantages and disadvantages, which should be weighed before a particular strategy is employed.

Table 3.1 Possible gene insertion strategies in *A. niger*

Gene insertion strategy	Description	Advantages/ Disadvantages	Author
Self replicating plasmid	Uses AMA1 replicator sequence from *A. nidulans* to obtain non-integrative gene expression.	High transformation efficiency **/ Unstable** expression, with rapid plasmid loss	(Aleksenko and Clutterbuck, 1997) (Storms *et al.*, 2005) (Verdoes *et al.*, 1994)
Co-transformation *(Random insertion)*	Using a selectable marker containing plasmid along with the gene of interest containing plasmid for gene integrations or deletions.	Rapid implementation, high copy number possible **/ Extensive** transformant screening needed	(Goosen *et al.*, 1989) (Punt *et al.*, 1987)
Marker bearing plasmid, with a heterologous gene expression or gene deletion cassette *(Random or targeted insertion)*	A fungal transformation marker and heterologous gene are inserted in the same locus. Deletions or targeted integration obtained by using homologous up- and downstream targeting sequences	Less screening required than for co-transformation. **/ More** elaborate cloning required, slower implementation	(Goosen *et al.*, 1987)
Complementation of non-functional native auxotrophic gene *(Targeted insertion)*	Use *pyrG* to compliment a *pyrG* auxotrophic mutant. Results in integration at *pyrG* locus	Directed integration of heterologous gene as a single copy / Difficult to insert multiple copies	(van Hartingsveldt *et al.*, 1987)

Gene insertion strategy	Description	Advantages/ Disadvantages	Author
Linear DNA gene-targeting substrate *(Random or targeted insertion)*	Created either through cloning, or multiple pcr fusions. Contains a fully functional marker, flanked by up- and downstream targeting sequences.	Higher transformation frequency than for circular plasmids **/ High** number of ectopic integrations when attempting deletions	(Pedersen H. *et al.*, 2000)
Bi-partite gene-targeting substrate *(Targeted insertion)*	Use two gene-targeting fragments, each consisting of a targeting sequence and either a N-terminal non-functional 2/3 or C-terminal non-functional 2/3 of a marker gene. The marker is reconstituted *in vivo* through homologous recombination, leading to higher targeting efficiencies. (see chapter 8)	Improved gene targeting percentage over linear substrate, pcr used to create substrate instead of cloning used in linear substrates, possible high throughput aplication **/ Lower** number of transformants obtained than from the single linear substrates approach	*S. cerevisiae*: (Erdeniz *et al.*, 1997) *A. nidulans*: (Michael L.Nielsen *et al.*, 2005) *A. niger*: This study

Targeted and random integration strategies are limited by the number of markers available for transformation. Auxotrophic markers (for example a pyrimadine auxotrophy) either have to be present or have to be introduced in a strain (many *A.* niger wild type (N402) auxotrophic mutants are available from the Fungal Genetics Stock Center: http://www.fgsc.net/niger.html), while dominant markers (such as antibiotic resistance) can be used without first manipulating the parental strain. Only a limited number of markers are available for *A. niger,* some of which are listed in table 3.2, and this is often a limiting factor when implementing a strain improvement strategy. This is a general problem in all organisms, but for some (*A. nidulans* and *S. cerevisiae*) advanced strategies have been developed allowing the recycling of a marker. Using a recyclable marker enables the repeated manipulation of a microbial genome with minimal effort. The attributes of a recyclable marker are: the ability to select as well as counter select for the marker, also the presence of two identical direct repeats flanking it. The recombination of the two direct repeats allows the marker to be looped out during counter selection.

Table 3.2 Fungal transformation markers available in *A. niger*

Selectable marker	Selection	Counter selection	Author
pyrG (A. niger) *pyrG* (A. fumigatus)	Cures pyrimidine auxotrophy Using *A. fumigatus pyrG* results in higher transformation efficiencies than native *A. niger pyrG*.	5-FOA	(Goosen *et al.*, 1987)
PgpdA-sC-trpC (A. niger)	Restores growth on sulfate only sulphur source media for sC^- (adenosine triphosphate sulfurylase) strains	Selenate	(Varadarajalu and Punekar, 2005) (Buxton *et al.*, 1989)
amdS (A. nidulans)	Dominant selectable marker. Enhances growth on acetamide as sole nitrogen source relative to inserted copy number	Amide	(Kelly and Hynes, 1985)
niaD (A. niger)	Enhanced growth on nitrate as sole nitrogen source	No	(Campbell *et al.*, 1989)
argB (A. nidulans)	Cures arginine auxotrophy	No	(Buxton *et al.*, 1985)
trpC (A niger)	Cures tryptophan auxotrophy	No	(Goosen *et al.*, 1989)
PgpdA-hph-trpC (E. coli)	Dominant marker. Confers hygromycin resistance	No	(Punt *et al.*, 1987)
oliC3 (A. niger)	Semi-dominant marker Confers oligomycin resistance	No	(Tolsters A., 2003)
pro (A. niger)	Cures tryptophan auxotrophy	No	(Swart *et al.*, 1992)
nicB (A. niger)	Cures a Nic⁻ mutant	No	(Verdoes *et al.*, 1994)

Once the transformation strategy has been decided, it is also important to select a transformation method, which will result in the most efficient transformation for your strategy. The most widely used transformation method in *A. niger* consists of making protoplasts using cell wall degrading enzymes, followed by addition of DNA in a transformation buffer and subsequent plating on, or in osmoticly stabilized selective media. This method works well for random gene insertions, as well as for gene targeting. The germling method has been reported to result in drastically more efficient transformations for random integration strategies in *A. nidulans*, although it has not yet

been proven to improve targeted integrations (Koukaki *et al.*, 2003). This method has not been attempted in a published work with *A. niger*, but it appears to be a promising method, although the question on gene-targeting efficiency would first have to be addressed. A more unconventional approach, atleast for filamentous fungi, has been reported by Ozeki *et al.* 1994. Here, transformation of intact *A. niger* hyphae was achieved through electroporation, a technique widely used on bacteria and yeast, but unfortunately no other papers could be found employing this technique. A list of the discussed transformation techniques, along with reported transformation efficiencies, can be seen in table 3.3.

Table 3.3 Fungal transformation methods

Transformation method		Author
Electroporation of intact *A. niger* hyphae	Electroporation of germinating conidia, transformation efficiency can be increased 2 fold by using cell wall degrading enzymes. 1.2 colonies or 100 colonies per µg integrative vector or µg plasmid DNA, respectively, were obtained.	(Ozeki *et al.*, 1994)
Protoplast method (most commonly used in *A. niger)*	Fresh mycelia are treated with a cell-wall degrading enzyme in an osmoticly stabilized buffer. The resulting protoplasts are then washed, concentrated, and transformed with the desired DNA substrate in a transformation buffer.	(Johnstone *et al.*, 1985)
Improvement on Protoplast method	The addition of dithiothreitol improves the transformation efficiency of *A. nidulans*, and should have a similar effect for *A. niger*.	(Dawe *et al.*, 2000)
Germling method (*A. nidulans)*	It was found that protoplasts prepared from germinating spores, at the stage where the germtube emerges, results in a 2-8 fold transformation efficiency improvement over normal protoplasting method, depending on the employed plasmid. Also significantly faster then normal protoplasting method.	(Koukaki *et al.*, 2003)

A final problem, which is hard to avoid, involves the pleiotropic effects of a heterologous, or native, auxotrophic curing gene being inserted in a non-native genomic locus during gene targeting. It is well known that different genomic loci have different levels of basal expression. This implies that the position of the auxotrophic marker gene in the genome effects the level of its expression. This altering of expression level could then affect the strain in unexpected and production effecting ways. Restoring the native activity of an auxotrophic marker is especially important for industrial applications, where years of strain improvement programs have resulted in very good producers, which must be altered as little as possible so as not to negatively influence their productivity or yield. This problem will be addressed in chapter 8 of this thesis, where the concept of using a reversible native auxotrophic marker will be introduced.

The advent of the post genomic era for *A. niger* will reveal many opportunities for advanced strain improvement, but if full advantage is to be taken it will surely be essential to develop more advanced gene manipulation strategies.

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Chapter 4: An introduction to physiological characterization using continuous fermentation

4.1 Introduction

There are several advantages of employing continuous fermentations to characterize and compare strains. The ability to achieve and maintain a reproducible, strain specific, steady state, allows for extremely accurate comparisons to be made between different strains. Reductions in the effects of different growth-rates can also be achieved, as the growth-rate is set through the dilution rate. Hereby it is possible to study the influence of a single operational parameter and with all other parameters kept constant, which greatly simplifies the interpretation of experimental results.

Continuous fermentations have been successfully employed in the study of bacteria and yeast mutants, but it is considerably more difficult to use continuous fermentations when filamentous fungi, such as *A. niger*, are studied. Several studies have reported the effects of different pH levels (Mainwaring *et al.*, 1999), nitrogen sources (Swift *et al.*, 2000), media components ((Carlsen and Nielsen, 2001); (Pedersen *et al.*, 2000)) and strains (Withers *et al.*, 1998) on enzyme production by *A. niger*. The advantages of using continuous fermentations in the study of enzyme producing filamentous fungi, was demonstrated by the discovery that α-amalyase production was both repressed and induced by glucose (Carlsen and Nielsen, 2001). This discovery could not have been made using batch fermentation experiments, as too many other factors, especially the changing glucose concentration, would have masked the results.

Unfortunately, there have not been many recent studies in *A. niger* performed on organic acid production in continuous culture. The most recent article (McIntyre and McNeil, 1997), achieved an outlet concentration of 3-4g/L citrate, at a dilution rate of 0.035 h^{-1} and a sucrose concentration of 100g/L, in an 8L fermenter with the citrate overproducing *A. niger* A60 strain. The effects of dissolved CO_2 concentration on strain physiology could also be studied in detail. This study demonstrated the fundamental advantage of being able to study one variable at a time. It was demonstrated that batch fermentation studies exaggerated the effect of dissolved CO_2 on citrate production and strain physiology, because it was not only the concentration of CO_2, but also the growth stage at which the CO_2 was added, as well as several other parameters, which affected strain physiology (McIntyre and McNeil, 1998). An older study, dating from 1979, achieved an citrate production rate of 0.46g/L.h, with an inlet glucose concentration of 50g/L, in continuous culture using *A. foetidus* (Kristiansen B. and Sinclair C.G., 1979). There was also a recent study performed on the effects of media composition on organic acid production in *Pennicillium simpicissimum* (Gallmetzer *et al.*, 2002), this medium composition will be discussed later in this chapter. The major difference between most of the reported media compositions and a composition designed for organic acid production is the limiting nutrient, nitrogen or phosphate for organic acid production, and glucose for most other studies.

It is essential to have a homogeneous culture in order to obtain a steady-state, but unlike budding yeast, filamentous fungi can have several different growth morphologies. Among others, we find filamentous growth, growth in clumps and different sizes of pelleted growth. It can be very difficult to control the morphology,

as many factors play a role. For example, the genotype of the mutant strain being studied can have a big influence. Except for the differences between strains, the growth medium used, the limiting nutrient employed, several other factors are involved, some of which will be discussed in more detail in this chapter.

4.2 Problems encountered in this study

During the course of this investigation several problems were encountered: wall growth, sporulation on the walls, and growth on internal parts (see figure 4.2), which led to biomass wash-out as can be seen in figure 4.1 and 4.2.
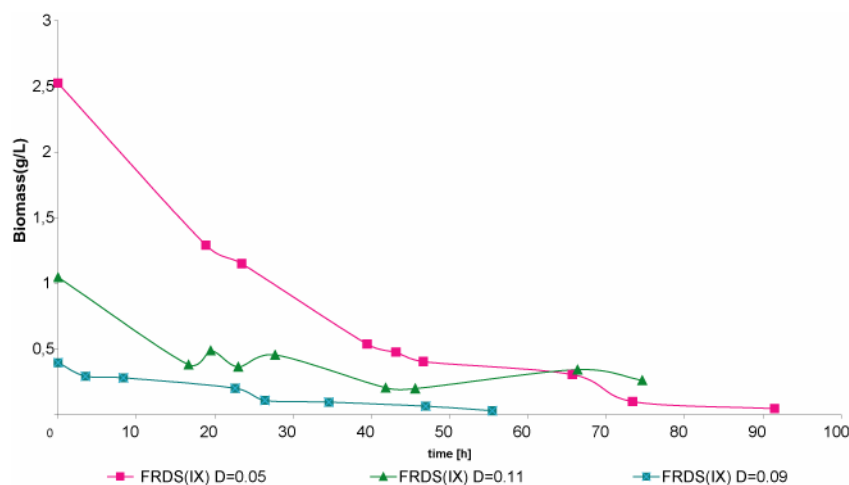
Figure 4.1 : Biomass plotted vs. time, showing typical washout curves at different dilution rates. Time 0 indicates when the fermentations were set to continuous.

Figure 4.2 Unwanted growth and sporulation in fermenters during continuous fermentation.

Attempts were made to remedy this situation by pulsing with higher stirring rates for 30 seconds at a time and by applying cooling to the headspace. Cooling the headspace did indeed decrease wall growth and sporulation, but pulsing the stirring appeared to have no effect on growth on internal parts. Different dilution rates appeared to have no positive effect on the wash-out of biomass, and it was therefore decided to change fermenter from BRAUN Biostat M to the newer BRAUN Biostat B. The growth on internal parts decreased slightly, but biomass wash-out was still occurring. Changing the media appeared to be the only option, and to this end *P. simplicicum* continuous fermentation succinate production media was chosen (Gallmetzer *et al.*, 2002)*,* but the wash-out problem persisted.

At this point it was decided to follow another published work, dealing with citrate production in continuous fermentation (Kristiansen B. and Sinclair C.G., 1979), they employed *A. foetidus* strain in their work, but it was still hoped that the change of media might solve the problems encountered. A major difference in this protocol was that, except for the changes in media composition, the stirring rate was much decreased, i.e. 300rpm instead of 900rpm.

4.3 Final results achieved in this study.

Switching to the Kristiansen media (Kristiansen B. and Sinclair C.G., 1979), coupled to headspace minimization and cooling, solved most of the problems associated with unwanted growth in the fermenter (such as in the headspace and on internal parts), but the morphology in the fermenter were still not completely homogeneous. At 300rpm large clumps were observed after a few days of fermentation, along with the appearance of very small pellets. It has to be noted that the Kristiansen media was designed to result in pelleted growth during the continuous fermentation, as this is the morphology of choice for citrate production. A total lack of Mn^{2+} in the medium, necessary for organic acid production, is also known to result in a pelleted morphology (Mattey M., 1992).

To remedy this we increased the stirring rate to 500rpm, which appeared to decrease the clump size, although the tiny (less than 2mm) pellets persisted. It was decided at this point to continue with the fermentation to see if a steady state could be reached and maintained for several residence times.
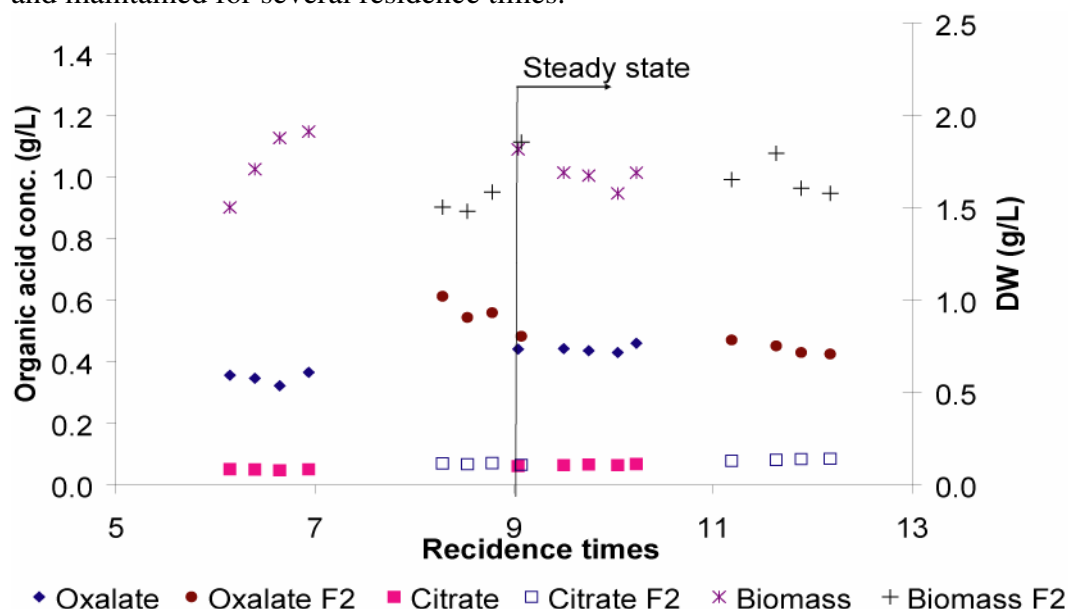
Figure 4.3 Biomass and metabolite curves vs. residence time for duplicate fermentations of strain N402 at a dilution rate 0.13h^{-1} , pH 3.5 and 500rpm. F2 in the legend indicates data from the duplicate fermentation.

Initial results were very encouraging, showing reproducible, steady biomass and metabolite profiles over several residence times (see figure 4.3). Two other strains were then investigated in continuous culture, one containing yeast *Frds1* behind a strong fungal gpdA promoter (strain Frds(IX)) and the other a gluconic acid negative mutant strain of the same background as N402 (NW131). Duplicate continuous fermentations were performed for N402 and Frds(IX) and intracellular metabolite samples were taken with the aim of performing an in-depth study of the effects of the glucose oxidase null-mutation and *Frds1* insertion (see chapter 5).

Unfortunately, when a rapid biomass sample from the wild-type fermentation was compared to a normal continuous sample, the actual biomass concentration in the fermenter was 2.8-3.2g/L, not 1.8-2g/L as was measured from the outlet during

cultivation (WT fermentations). This indicates that there is either, an accumulation of biomass in the fermenter, or fluctuations in the biomass outflow over short time scales (e.g. sampling time scale), resulting in either a more complex steady state than the one desired, or an inaccuracy in the final biomass calculations. Figure 4.4 demonstrates that there is a high likelihood of fluctuations in the biomass outflow being the reason for the problems with the biomass determinations. In the figure the last sampling point is circled, and it can be seen that this triplicate determination, taken in direct succession to each other, has a variation of 0.5g/L (2.3g/L to 2.8 g/L) between the three points. It would therefore appear that a normal steady state were achieved in the cultivations, as were confirmed by metabolite and CO_2 analysis, although the biomass determinations during the cultivations were prone to significant variations over short time scales.
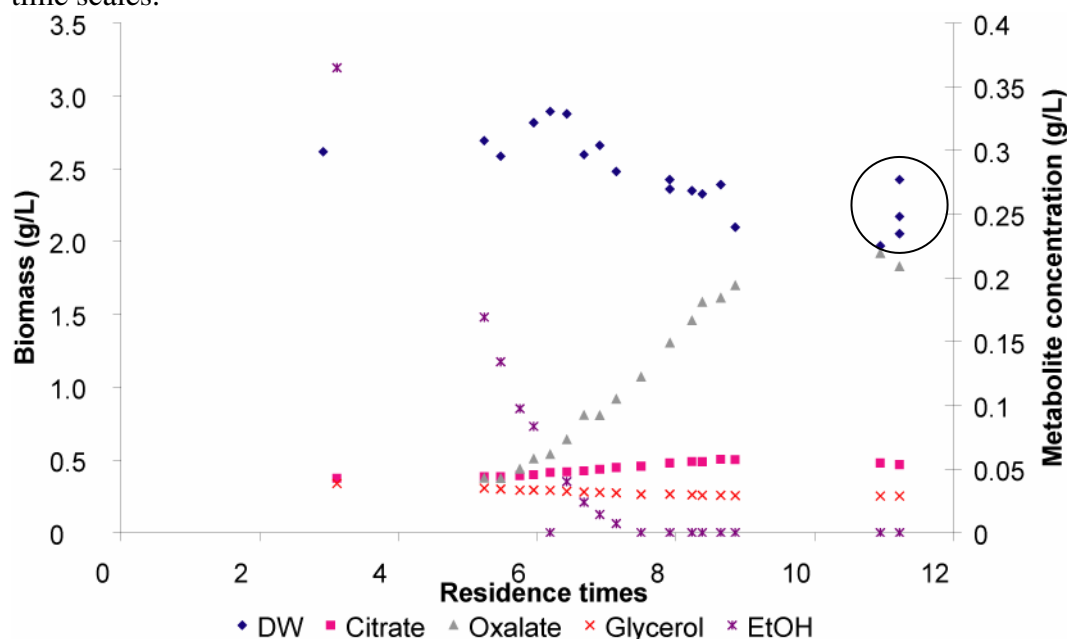
Figure 4.4. Biomass and metabolite production over time for the glucose oxidase negative *A. niger* strain. The final point, circled in the figure, consists of a triplicate measurement. This measurement illustrates the high variability in the outlet biomass concentration.

It was decided to use the simpler batch fermentation system after the detailed characterization of the wild-type and two mutant strains. The reasons the continuous fermentation approach was replaced with the batch fermentation system, were: The high rate of failed fermentations (biomass growth on internal parts, accompanied by washout, as well as variable morphology in some strains) and the time investment necessary to optimize the fermentation for each new strain used. Also a simple batch fermentation system was found to be sufficient for evaluation of the different strains constructed in this study, while the more advanced continuous fermentation system was found to be excellent for in-depth study and comparison of a small number of strains, as can be seen in the next chapter.

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Chapter 5: Physiological characterization of *A. niger* using continuous fermentation and intracellular metabolite profiling.

Introduction

Continuous fermentation is a powerful tool in the study of mutants created throughout a metabolic engineering project. The ability to control the specific growth-rate through the dilution rate permits mutants with dissimilar specific growth-rates to be accurately compared. The high level of reproducibility inherent in continuous fermentations allows for very accurate comparisons to be made between strains, and with a confidence in the results that enables the detection of very small effects resulting from gene insertions or deletions.

Several successful studies employing continuous fermentations to study enzyme production have been published, for example: effect of morphological mutants on enzyme production in chemostat cultures (Withers *et al.*, 1998), and effects of oxidative stress on protein hen egg white lysozyme production (Kreiner *et al.*, 2003). There are only a few recent studies that have been published on organic acid production in chemostat cultures, e.g. a study of *P. symplicissimum* under different nutrient limitations (Gallmetzer *et al.*, 2002), and citrate and gluconate production in *A. niger* A60 (McIntyre and McNeil, 1997). There are several reasons for the few studies on organic acid production in chemostat culture; the major organic acid of interest in *A. niger,* citrate, is not adequately produced in continuous cultures (Karaffa L. and Kubicek C.P., 2003). Also, organic acid production generally requires a very limiting trace metal composition for *A. niger*. Specifically, manganese has to be excluded, which leads to difficulties with growth morphologies as a lack of manganese encourages pellet formation [(Karaffa L. and Kubicek C.P., 2003), (Mattey M., 1992), (Ruijter G.J.G., Kubicek C.P., and Visser J., 02)], which is inconsistent with continuous fermentations.

In this paper the basis of organic acid production in *A. niger* was studied through detailed physiological characterizations of three strains with dissimilar organic acid production profiles. These were the wild-type N402 strain, a glucose oxidase mutant strain, and a strain overexpressing the yeast cytosolic soluble fumarate reductase gene (*frds1)* in the N402 background.

The *frds1* gene has been shown to irreversibly convert cytosolic fumarate to succinate in yeast, using $FADH_2$ as a co-factor (Arikawa *et al.*, 1998). *A. niger* N402 does not contain cytosolic fumarase or fumarate reductase activity [(Bercovitz *et al.*, 1990); (David H. *et al.*, 2003)], and the inserted *frds1* protein product would therefore need to use mitochondrially produced fumarate. The effect of $FADH_2$ consumption in the cytosol is also hard to predict as there are few reported enzymes in *A. niger* using this co-factor. The localised effects of the insertion were determined by intracellular metabolite profiling, while the systems effects of the co-factor usage were determined through physiological characterization.

The glucose oxidase mutant strain has been shown to be a good citrate producing strain under conducive conditions (Ruijter G.J. *et al.*, 1999). It was expected that there would be only minor differences between the glucose oxidase mutant and the WT, as the medium and operating condition employed for the continuous fermentations does not lead to gluconic acid (the product of glucose oxidase) formation. However, since the strain is the product of mutagenesis, a slightly different organic acid profile was still expected.

5.1 Materials and Methods

Fungal strains. All Aspergillus niger strains used in this study originates from the N402 reference strain. Strain NW131, a glucose oxidase negative mutant, was obtained from (Ruijter G.J. et al., 1999).

5.1.1 Plasmids. Manipulation of plasmid DNA and transformation of plasmids into *Escherichia coli* DH5α were carried out according to standard procedures (Sambrook, J. and Russell, D. W., (2001)). All plasmids were constructed as random genomic integrating vectors. The *Frds1* gene used in this study was placed between the *gpdA* promoter and the *trpC* terminator on the plasmid FRDS_A1. (Citrate production paper: chapter 7)

5.1.2 Fungal Transformation
PyrG- mutant A742 was transformed with plasmid pFRDS A1 containing the *Frds1* gene and *A. niger pyrG* as marker to create the strain Frds(IX). The transformation was carried out as described in Michael L.Nielsen *et al.*, 2005.

5.1.3 Media for strain development. *A. niger* strains were grown at 30°C on solid minimal media (MM) (Clutterbuck, A. J., (1974)) or on solid MM containing uracil and uridine for *pyr⁻* auxotrophic strains. Selection of transformants were performed either on MM media. Bacterial strains were grown at 37°C in LB medium with 100 μg ampicillin/mL.

5.1.4 Inoculum. The strains were stored at -80°C in Eppendorf tubes containing 0.01% Tween with 20% (v/v) glycerol. From the stock tubes, point inoculations with a toothpick on plates with selective media for plasmid bearing strains were used.

5.1.5 Medium for the continuous cultivations. The medium for the continuous cultivations had the following composition: 0.5g/L NH_4NO_3; 0.5g/L KH_2PO_4; 0.1g/L $MgSO_4\bullet 7H_2O$; 1 mL/L 1000 X trace element solution containing per litre: 0.037g/L $FeCl_3$, 0.06g/L $CuSO_4.5H_2O$, 0.1g/L $ZnSO_4.7H_2O$; 50 μL/L antifoam (Sigma, A-8436); and 23.5 g/L glucose (Kristiansen B. and Sinclair C.G., 1979).

5.1.6 Cultivation conditions. The cells were grown as aerobic continuous cultivations with nitrogen eventually being the growth-limiting component. The cultivations were carried out in well-controlled 2-litre Braun Biostat B bioreactors with working volumes of 2 litres. The bioreactors were equipped with two disk turbine impellers rotating at a maximum speed of 500 rpm. The pre-batches for the continuous fermentations had the same medium composition as for the continuous fermentation, but the initial pH was set to 2.5, agitation was set to 150rpm, and the initial aeration to 0.1 VVM. After germination, approximately 16 hours, the cultivation conditions were stepwise increased to the operating conditions: 500rpm, pH 3.5 and 1VVM. The batch fermentations were set to continuous mode in mid exponential, and allowed to reach steady-state. It was assumed steady-state was reached when the determined biomass, CO_2 and metabolite concentrations were stable over several residence times.

5.1.7 Cell mass determination. The cell mass concentration on a dry weight basis was determined by the use of nitrocellulose filters with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, Mich.). Initially, the filters were predried in a microwave oven at 150 W for 10 min., and then weighed. A known volume of cell culture was filtered, and the residue was washed with distilled water. Finally, the filter was dried in the microwave at 150 W for 15 min., and then weighed.

5.1.8 Analysis of extracellular metabolites. For determination of the extracellular metabolites, a sample was taken out of the bioreactor and immediately filtered through a 0.45 µm-pore-size cellulose acetate filter (Osmonics, Minnetonka, MN). The filtrate was frozen and kept at -20°C until analysis. Organic acids and glucose were separated on an Aminex HPX-87H column (Biorad, Hercules, Calif.) at 65°C, using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and detected refractometrically (Waters 410 Differential Refractometer Detector; Millipore Corp., Milford, Mass.).

5.1.9 Sampling and Analysis of intracellular metabolites. Intracellular metabolite samples were taken when the fermentation was determined to be in steady-state. The samples were rapidly taken and immediately quenched in -40°C MeOH, with a final concentration of 50% MeOH. The samples were then filtered and washed with 200-300ml of 50% MeOH. The whole filtration process was carried out in the -20°C cold room, using 50% MeOH precooled to -20°C. The protocol of Villas-Boas (Villas-Boas et al., 2003) was then followed, with minor changes. 1.5 times more buffer and methanol was used for extraction to facilitate better mixing during the 45min. shaking stage at -20°C. Also, 50mM triethanolamine was added to the MeOH used during quenching and washing, according to the method of Ruijter (Ruijter G.J. and Visser J., 1996) for filamentous fungi.

The intracellular metabolites were analysed on GC-MS after MCF derivitization according to the method of Vilas-Boas (Villas-Boas *et al.*, 2003).

5.2 Results

Three strains were investigated in continuous culture, the wild type (N402, refered to as WT), a strain of the WT background containing *S. cerevisiae Frds1* expressed behind a strong fungal gpdA promoter (strain Frds(IX)), and a glucose oxidase negative mutant strain of the same background as N402 ,NW131 (also referred to as Δgox). Figure 5.1 demonstrates the reproducibility and stability of the steady-state achieved with the WT. Duplicate continuous fermentations with a dilution rate of 0.125 ±0.005 were performed for WT and Frds(IX), as well as a single fermentation of Δgox.
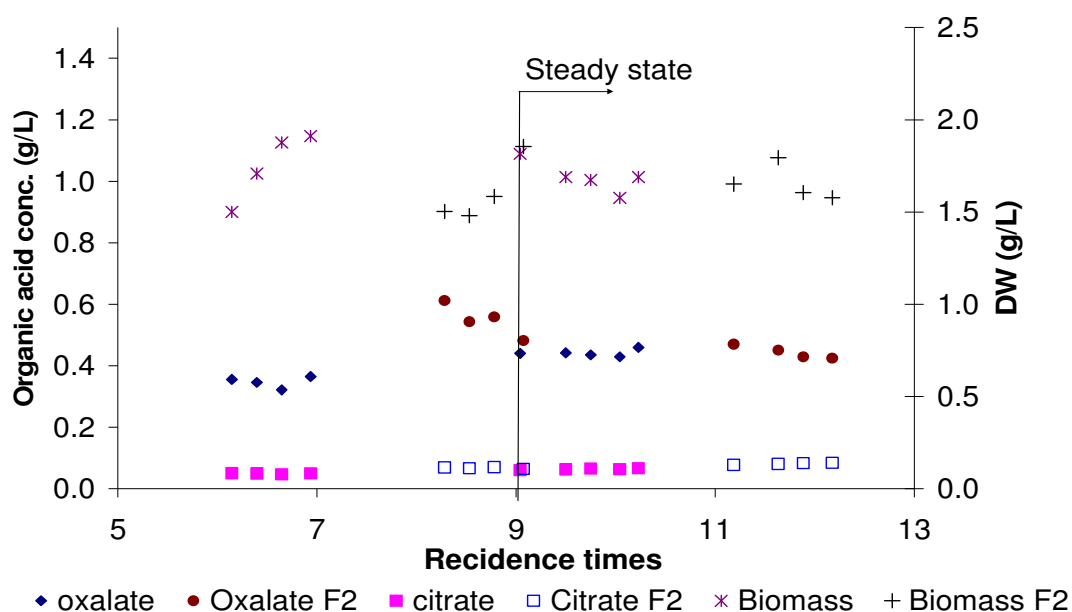
Figure 5.1. Biomass and metabolite curves vs. residence time for duplicate fermentations of strain N402 at a dilution rate 0.13h^{-1} , pH 3.5 and 500rpm. F2 in the legend indicates data from the duplicate fermentation.

The comparative productivities for dry weight, CO_2 and extracellular metabolites detected by HPLC from all the steady-states can be seen in figure 5.2. The most apparent changes between the wild type and the mutant strains are the almost 50% decrease in oxalate production and the significant increase in CO_2 production for the mutant strains. A significant increase in citrate productivity is also apparent for Frds(IX), while the Δgox strain has an increased biomass productivity.
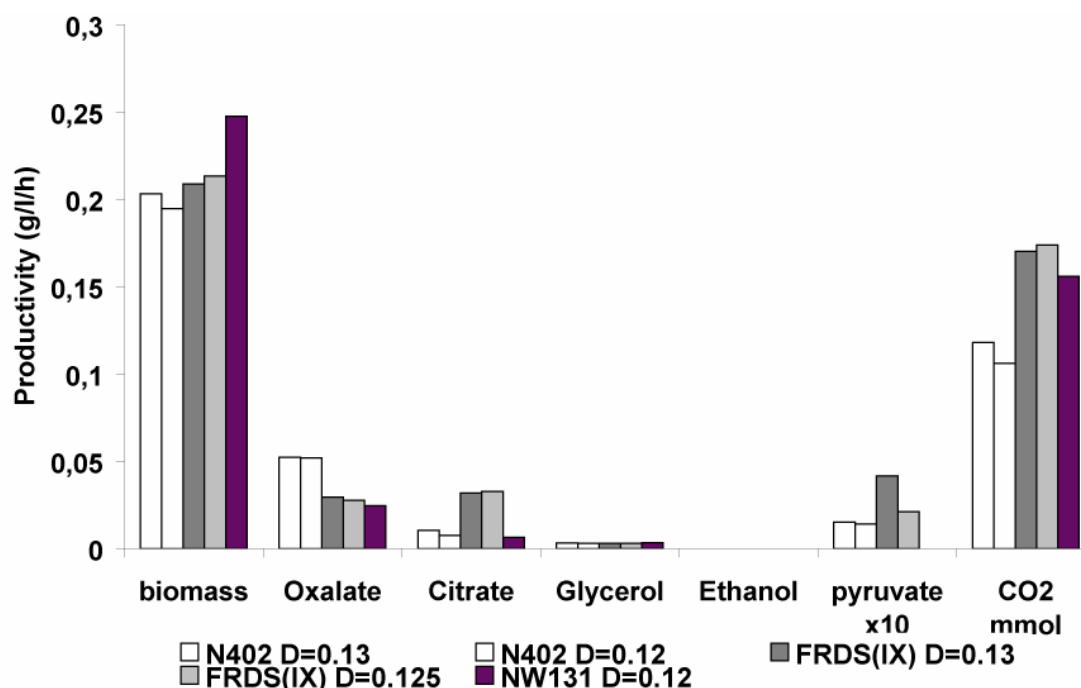
Figure 5.2. The productivities of the extracellular metabolite detected by HPLC, as well as CO_2 and dry weight measurements are represented for all the fermentations included in this study. The dilution rate is indicated as D in each case.

Table 5.1. Physiological parameters

Strain	Ysx (gDw / g Glc)	Glucose uptake rate(g / gDw.h^{-1})	Residual glucose (g / L)	RQ	Oxygen consumption (mmol/L.min)
N402 (WT)	0.48±0.025	0.26±0.02	20.4±0.1	0.095±0.05	15.5±0.3
Frds(IX)	0.46±0.05	0.32±0.01	20.1±0.4	0.135±0.05	16.2±0.3
Δgox (NW131)	0.61	0.29	20.4	0.19	14.5

Intracellular metabolite samples were taken from one fermentation for all three sets of fermentations, with the aim of performing an in-depth study of the effects of the glucose oxidase negative mutation, and the *Frds1* insertion. The results from the intracellular metabolite profiling can be observed in figure 5.3 and 5.4. Triplicate samples were analysed for Δgox and four samples for Frds(IX), while only a single sample was obtained for the WT. From figure 5.3 it can be seen that the WT has higher intracellular concentrations of all amino acids and organic acids measured, except for lactate, succinate and fumarate.
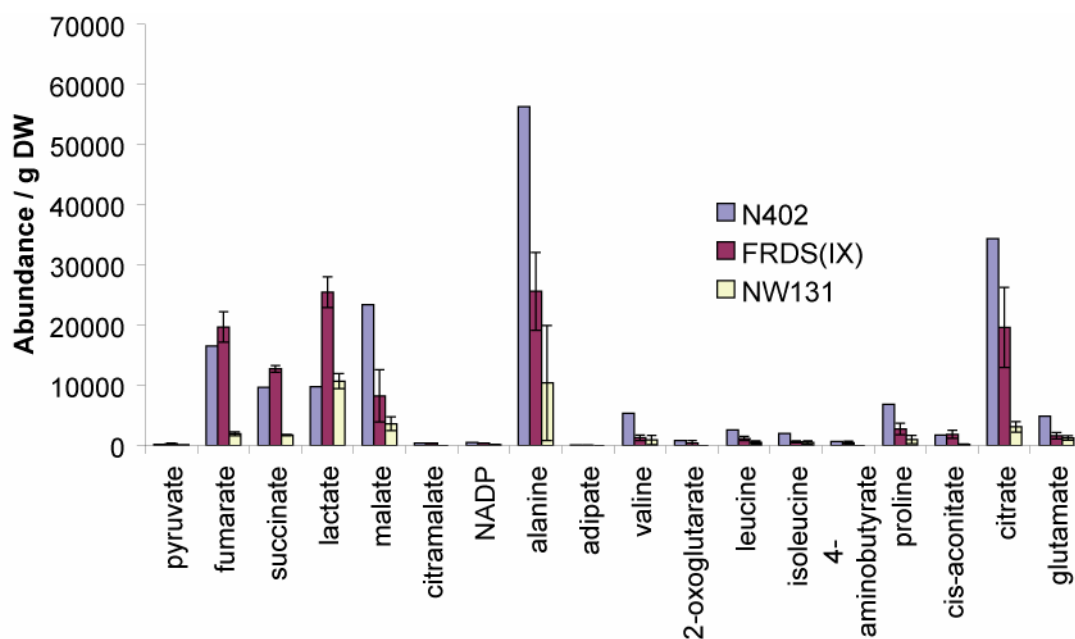
Figure 5.3. Measured abundances for intracellular metabolite profiling using MCF derivitisation followed by GC-MS analysis.

The concentrations of the organic acids of most interest were determined in mM, using calibration curves, and are displayed in figure 5.4. It is interesting to note that more lactate was present in Δgox than in the WT, even though all the other organic acid concentrations were lower. Comparing the WT and Frds(IX), we see that Frds(IX) has higher intracellular organic acid concentrations for succinate, lactate and possibly fumarate.
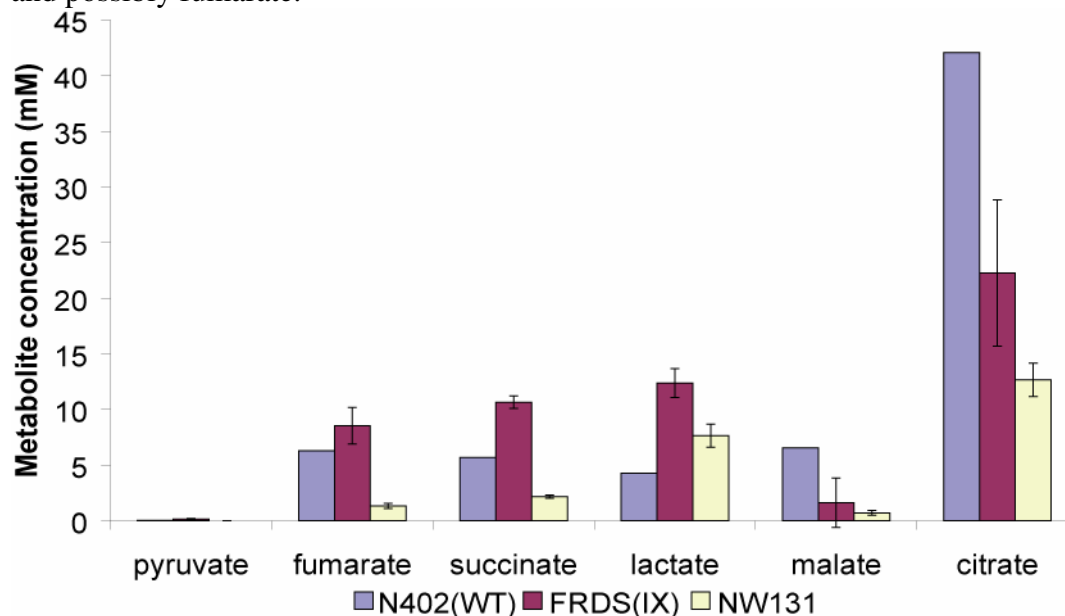
Figure 5.4. Intracellular concentrations for the main organic acids detected by GC-MS

5.3 Discussion

The differences in organic acid production, as well as the relations between the intracellular metabolites, were analysed in detail. The aim of the analysis was to determine the underlying mechanisms of organic acid production under these conditions. The large differences observed for oxalate production is particularly interesting as oxalate is a toxic and problematic by-product in many processes.

5.3.1 Decreased oxalate production

The decrease in oxalate production observed for the two mutant strains are not reflected in the glucose uptake rates (see table 5.1). Although, the decrease in production is accompanied by an increased glucose uptake rate for Frds(IX), Δgox is not significantly affected. This indicates that the reduction in oxalate production is not caused by a pleiotropic effect of gene insertion, or mutation, directly negatively affecting the uptake of carbon or the production of products, but instead is caused by the redirection of flux within the cell caused by the specific insertion and mutation being studied. From table 5.1 and figure 5.3, it can be seen that the main effects of the *frds1* gene insertion were increased organic acid production (citrate, fumarate and pyruvate) and CO_2 production, while the Δgox mutant had increased CO_2 and biomass yields.

The increased CO_2 production rate for the Δgox strain appears to be linked to the highly increased biomass yield. This would suggest increased pentose phosphate (PP) pathway activity, which would also lead to increased CO_2 production. The assumption that the excess CO_2 was produced by the PP pathway can also be supported by the decreased oxygen consumption rate (14.5 vs. 15.5mmol/l.h for the wild type), which points to lower TCA-cycle activity. Diverting the carbon flux to a greater extent into the pentose phosphate pathway and biomass formation, would decrease the flux towards pyruvate, and would therefore be responsible for the decreased oxalate production rate observed.

Increased production of extracellular citrate, coupled to the increased CO_2 production rate and oxygen consumption rate, suggests an increased TCA-cycle activity in strain Frds(IX). This increased TCA-cycle activity would explain the decreased oxalate production rate, as more carbon would be directed into the TCA cycle (through the import of pyruvate), and away from oxaloacetate (OAA), the substrate for oxalate production. There are at least two ways in which the insertion of *frds1* could result in increased TCA-cycle activity: Creating an increased cytosolic demand for fumarate, or through the effects on redox through $FADH_2$ consumption.

5.3.2 Effects of the *frds1* insertion on intracellular metabolites

At this time no direct connection between cytosolic and mitochondrial $FADH_2$ pools has been reported in the literature, although it has been shown in *S. cerevisiae* that *frds1* is required for $NADH_2$ reoxidation under anaerobic conditions (Arikawa *et al.*, 1998). It has also been postulated that fumarate respiration through the reductive arm of the TCA cycle was responsible for maintaining the $NADH_2$/NAD+ redox

balance under anaerobic condition in *P. symplicissimum* (Gallmetzer *et al.*, 2002), which also led to higher succinate production. Therefore, a likely mode of action for increasing TCA-cycle activity is the shuttle mechanisms required for $NADH_2/NAD^+$ transfer between the cytosolic and mitochondria, which is well known in *A. niger*. The decrease in ethanol production compared to the WT, caused by the insertion of *frds1* under oxygen limiting conditions, supports the assumption of an effect on the $NADH/NAD^+$ ratio. (See chapter 6)

Interestingly, when the intracellular metabolites were studied, it was seen that it was only succinate and lactate, which were significantly increased in Frds(IX). Fumarate and pyruvate showed only a slight increase, cis-aconitate had the same concentration, while citrate, alpha-ketogluterate and malate were all higher in the WT. On closer examination, it can therefore be seen that the insertion of *frds1* specifically affects succinate and the pyruvate node (from where oxalate and lactate, as well as the TCA intermediates, originates). It was most likely cytosolic succinate, which was affected, since frds1p is a cytosolic enzyme.

5.3.3 Increased intracellular lactate concentrations

The effect of the gene insertion and the mutation on lactate could be related to redox, as lactate dehydrogenase (converting pyruvate to lactate) is a NADH dependent enzyme. Intracellular lactate was determined to have a correlation with the intracellular ratio of fumarate/malate for the three strains (R^2 of 1 for an exponential fit), and inversely proportional to the intracellular malate concentration. The inverse relationship of lacate to malate corresponds to the substrate competition between pyruvate carboxylase and lactate dehydrogenase, as OAA (the product of pyruvate carboxylase) is the substrate of malate dehydrogenase.

In conclusion, the insertion of *frds1* specifically influences the intracellular concentration of succinate and lactate, and to a lesser extent fumarate. It also has a major effect on extracellular oxalate production. The effect on intracellular fumarate and succinate is an indication of the direct fumarate reductase activity of *frds1*, while its effect on oxalate and lactate appears to be redox linked. The goxA6 mutation in the Δgox strain, appears to have the unexpected effect of significantly increased pentose phosphate pathway activity and in so doing increased the yield of biomass on glucose to a very high value of 0.6 g/g glucose.

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Chapter 6: Efforts towards engineering a succinate producing *A. niger* strain

<u>Introduction</u>

A. niger very efficiently produces citrate, and it is therefore used industrially for citrate production where it has been possible to obtain both very high titers and high productivities. Diverting this enormous production potential towards succinate would be of great industrial and environmental value. Currently succinate trades for approximately $4-6 per kilogram, while the bulk price for citrate is $0.5 per kilogram, which should make it reasonable to assume that an efficient succinate biological production process could favorably compete with the current petrochemical production routes. Succinate also represents an environmentally friendly option to currently employed benzene-derived C-4 compounds, such as certain adhesives, solvents, ext. Besides the industrial interests in biological succinate production, metabolic engineering strategies involved in enabling the redirection of carbon flux towards succinate would also represent an illustration on how it could be possible to re-direct carbon fluxes within the TCA cycle of *A. niger*, something which has not been shown possible before. This may allow the application of *A. niger* for the production of other similar products.

Succinate is not naturally produced by *A. niger* in any meaningful amount, whereas much better producers can be found among certain rumen bacteria (*Anaerobiospirillum succiniproducens, E. coli, ext)*, and to a lesser degree yeast (*S. cerevisiae*). Currently the reported production characteristics of the bacterial succinate producers are yields higher than 0.9 mol/mol glucose (Lin *et al.*, 2005b), titers between 80-120g/L (Guettler M. *et al.*, 1996), and fermentation times in the range of 48 hours (Guettler M. *et al.*, 1996). The major current advantage of *A. niger* compared with hyperproducing bacteria is, however, its ability to grow and produce at very low pH (pH 2), and the acid can therefore be produced in it's disassociated form. This prevents the problems associated with recovery of the acid, and disposal of the waste precipitate, which are formed when bacterial fermentations are performed at higher pH. Although it is certain that chemical engineering solutions to the precipitation problem will, in time, be found, it will surely also increase production costs and might therefore still allow an *A. niger* based process to be more competitive. The extremely large production base of *A. niger* for citrate production, and the technical expertise that goes along with this, also provides a competitive platform for using *A. niger* for organic acid production compared with competing microbial systems.

6.1 Metabolic engineering strategies employed for increased succinate production in various strains

Several attempts have been made at improving the natural production capabilities of succinate producing bacteria such as *E. coli* ((Lin *et al.*, 2005a); (Lin *et al.*, 2005c)) and *W. succiniproducens* (Guettler M. *et al.*, 1996). These strategies mostly focused on relieving glucose repression of the glyoxalate bypass, increasing the PEP carboxylase activity, deleting succinate dehydrogenase, ext. (see Table 6.1). *E. coli* has already been engineered to achieve very high aerobic succinate production rates, and the maximum theoretic yield of 1mol / mol glucose has been achieved under anaerobic conditions. A yield of 1.6 mol /mol glucose has also been reported under aerobic conditions (Sanchez *et al.*, 2005).

In eukaryotes the effects of compartmentalization plays a very important role. In *S. cerevisiae* Arikawa *et al.* (Arikawa *et. al.* 1999b) studied the effects of single and multiple deletions of TCA-cycle genes on succinate production, and found that the main route to succinate production was through the reductive branch of the TCA-cycle. This includes the cytosolic steps of pyruvate carboxylase and malate dehydrogenase, and the mitochondrial steps of fumarase and fumarate reductase. It has also been proven that the cytosolic fumarate reductase (*frds1*) is responsible for the biggest fraction of the produced succinate under anaerobic conditions in *S. cerevisiae*.

The production of succinate by filamentous fungi has only been studied in a limited number of cases. Gallmetzer et al. (Gallmetzer *et al.*, 2002) did media optimization to achieve high yields and productivies in *P. simplisicum*. From their research it could be deduced that *P. simplisicum* has an altered organic acid transporter compared to *A. niger*, as it seems to preferentially export succinate over citrate. The reason for succinate production under the studied conditions was concluded to be an energy spilling mechanism, similar to citrate production in *A. niger* (Gallmetzer *et al.*, 2002). It has also been shown that *A. flavus* produces succinate as a by-product in a malate production process, but the ability of *A. flavus* to produce mycotoxins makes it unsuitable for industrial applications.

A modeling approach was followed by David et al (David H. *et al.*, 2003), employing a detailed stoichiometric model of the central carbon metabolism to do an *in silico* deletion study in *A. niger*. The study resulted in the prediction of an increased succinate yield in a double deletion mutant (pyruvate decarboxylase and ATP:citrate OAA lyase) under oxygen limiting conditions, but the results have not yet been tested experimentally.

Table 6.1. Review of strategies employed for increased succinate production

Strain	Methodology	Result	Author
E. coli	Deletion of sdh sdhAB, (ackA-pta), poxB, iclR (relieves repression of glyoxalate bypass), and ptsG	0.91 mol/mol Yield Aerobic chemostat d=0.1 h^{-1}	(Lin *et al.*, 2005a)
E. coli	Overexpression of a mutant Sorghum pepc. Deletion of sdh sdhAB, (ackA-pta), poxB, iclR), and ptsG	Achieve a yield of 1 mol / mol glucose in aerobic batch fermentation, with increased productivity.	(Lin *et al.*, 2005b).
E. coli	Deactivation of adhE, ldhA and ack-pta. As well as , iclR	Yield 1.6 mol/mol Productivity 10 mM/h Anaerobic Batch	(Sanchez *et al.*, 2005)
E. coli	PEPC and pyruvate carboxylase overexpression, coupled to deletion of by-product pathways.	Increased succinate production	(Lin *et al.*, 2005c)
W. succuniproducens	Strain isolation from cattle rumen, isolation of sodium monofluoroacetate resistant mutants.	80-110g/L succinate titer	(Guettler M. *et al.*, 1996)
S. cerevisiae	Uracil auxotrophy or Deletion of *Osm1* or Frds1&SDH or SDH or SDH&Fum	Higher succinate production 1.5 x increase 2.7 x increase 1.6 x increase 2.6 x increase	(Arikawa *et al.*, 1999b)
P simplicissimum	Media optimization	Increased succinate production	(Gallmetzer *et al.*, 2002)
A. flavus	Media optimization with, high $CaCO_3$ concentration, leads to high pyruvate carboxylase activity	High malate production, succinate and fumarate produced as by-products	(Peleg Y. *et al.*, 1988)
A .niger	*In silico* deletion of pyruvate decarboxylase and ATP:Citrate OAA lyase	Predicted improved succinate production under oxygen limiting conditions	(David H. *et al.*, 2003)

Unfortunately, extensive genetic engineering of *A. niger,* such as multiple deletions of as-yet un-identified genes, was not realistically attainable at the start of this Ph.D., and the more simple approach of gene-insertions was therefore followed. The necessary tools for multiple deletions were also developed (see chapter 8), and the release of the *A. niger*

genome in 2006 will hopefully soon allow for more advanced, model based, metabolic engineering approaches to be employed.

6.2 Insertion of an alternative cytosolic pathway towards increased succinate production in A. niger

In bacteria and yeast, fumarate respiration is the main route towards succinate production. *A. niger* does not have any cytosolic fumarase or fumarate reductase enzymatic activity (David H. *et al.*, 2003) and succinate therefore has to be produced by the citric acid cycle, via the glyoxalate bypass, or the gaba-shunt. The TCA cycle is extremely well regulated, and any succinate produced would have to be exported from the mitochondria first. Also, the alternative pathway through the glyoxalate cycle is repressed under carbon repressive conditions, and very little is known on the regulation of the GABA-shunt. It was therefore decided to insert a cyctosolic pathway towards succinate production, by inserting a cytosolic fumarase (encoded by *Fum1* or *FumR*) and a cytosolic fumarase reductase (encoded by *Frds1)*. The details of the strain construction process can be seen in Chapter 7.

Studies in *S. cerevisiae* have shown that overexpression of *S. cerevisiae* cytosolic fumarase (*fum1*) leads to the excretion of citrate. The reason for this was proposed to be the 17 fold higher affinity of *fum1* for fumarate over malate coupled to the action of the *S. cerevisiae* mitochondrial malate-citrate antiporter (Pines *et al.*, 1996),. Succinate is produced mainly through this pathway (Arikawa *et al.*, 1999a)in *S. cerevisiae* under anaerobic conditions, and the expression of *fum1* in conjunction with *Frds1* was therefore still expected to lead to higher succinate production in *A. niger*. However, since the overexpression of *Fum1* in yeast raised some questions concerning the possible outcomes in *A. niger*, a second fumarase was cloned into *A. niger*. The second fumarase (*FumR*) originated in *Rhyzopus oryzae* NRRL 1526, a strain specifically used for fumarate production. Higher expression levels of *FumR* in *R. oryzae* has been shown to lead to higher fumarate productivities (Friedberg *et al.*, 1995), and it was therefore hoped that it could provide a more favorable target for creating a cytosolic pathway towards succinate production.

The single gene of both *fumR* and *fum1* have been reported to result in a cytosolic and mitochondrial enzyme product, which necessitated the truncation of the mitochondrial targeting sequence for both enzymes to ensure that the enzyme activities were targeted to the cytosol. Both *Fum1* and *FumR*[1] were therefore cloned as truncated versions, lacking the initial 17 and 15 amino acids, respectively, which ensure cytosolic targeting of the enzymes (see chapter 7). The truncation has been shown to result in purely cytosolic activity in *S. cerevisiae* ((Wu and Tzagoloff, 1987); (Peleg *et al.*, 1990)), while in *R. oryzae* the mitochondrial targeting sequence had been identified (Friedberg *et al.*, 1995), but the effect of a truncation had not been tested.

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[1] The truncated version of *fumR* and *fum1* will be referred to as *fumRs* and *fum1s* in the rest of the text.

The *Frds1* gene has been shown to irreversibly convert cytosolic fumarate to succinate, using non-covalently bound FAD as co-factor (Muratsubaki and Katsume, 1985). A possible problem with this approach is the lack of detailed knowledge of the possible $FADH_2$ contributing pathways active in *A. niger* under glucose repressing conditions (see Table 6.2 for reported cytosolic $FADH_2$ production or consumption pathways in *A. niger* (David *et al*. 2003)). It is also reported that $FADH_2$ is oxygen sensitive, which might lead to a lack of this co-factor during aerobic fermentations.

Table 6.2. Reported reactions utilizing either FAD^+ or $FADH_2$ in the cytosol. (David H. *et al.*, 2003)

Enzyme and reaction	Co-factor
D-Glucose + FAD_mit <-> D-Glucono-1,5-lactone + FADH2_mit (1.1.99.10)	FAD^+
Glycerol 3-phosphate + FAD_mit <-> Glycerone phosphate + FADH2_mit (1.1.99.5)	FAD^+
Glycerone phosphate + FADH2_mit + H2O -> Glycerol + FAD_mit + Orthophosphate	$FADH_2$
Lipid formation	$FADH_2$

6.3 Physiological strain characterization through various batch fermentation strategies

The strains created throughout this study were characterized under different conditions in an evolving process to find both better fermentation strategies, and better strains for succinate production. Changing the medium composition was initially necessitated by the very large impact on the organic acid production profile reported for several trace elements (Mattey M., 1992). The observation of large quantities of by-product for a specific mutant strain also led to changes in further medium compositions employed.

The initial production medium was chosen for its published ability to lead to malate production in *A. flavus, A. paraciticus* and *A. oryzae* ((Abe S. *et al.*, 1962), (Peleg Y. *et al.*, 1988)). Some changes were, however, implemented. The changes were: no $CaCO_3$ was added (vs. 2-10% in the original Peleg media (Peleg Y. *et al.*, 1988), this was done to avoid the problems encountered in fermenting a slurry, and analyzing precipitated organic acids), and a lower pH of 3.5 vs. 5-7 for the reported medium. The lower pH was chosen as gluconic acid is reported not to be produced at low pH values (Mattey, 1992). Figure 6.1 shows the results for the WT, an *Frds1* containing mutant (Frds(V)) and a glucose oxidase negative mutant strain (NW131, (Ruijter G.J. *et al.*, 1999)).
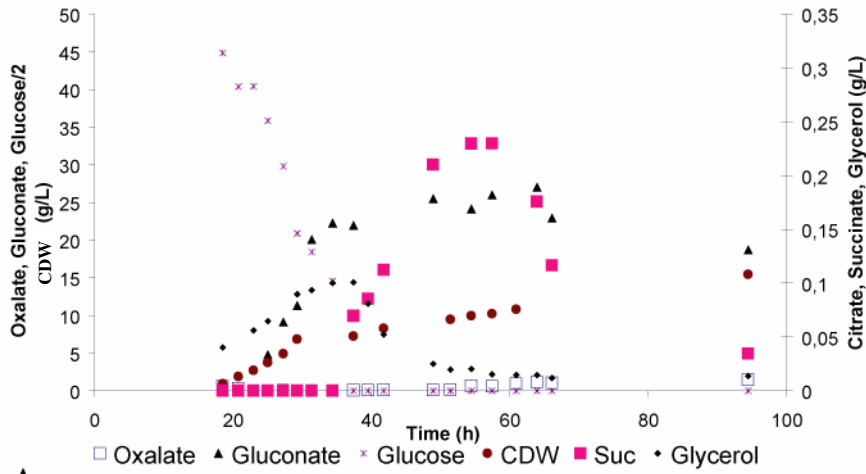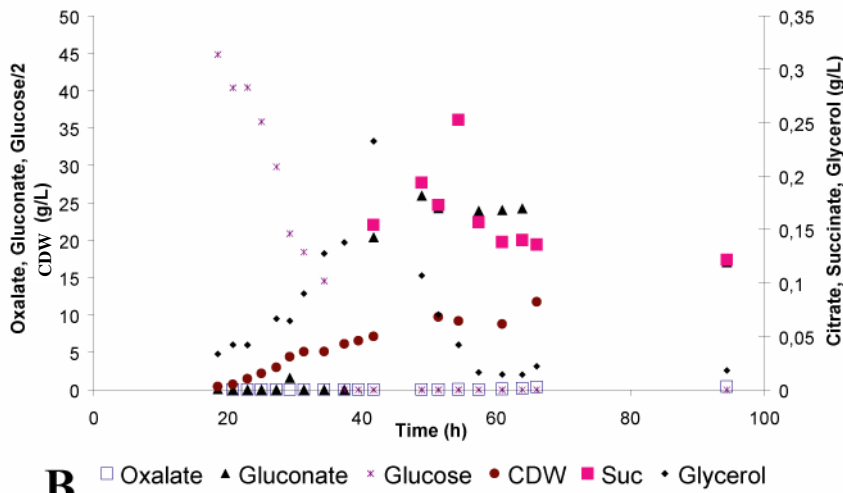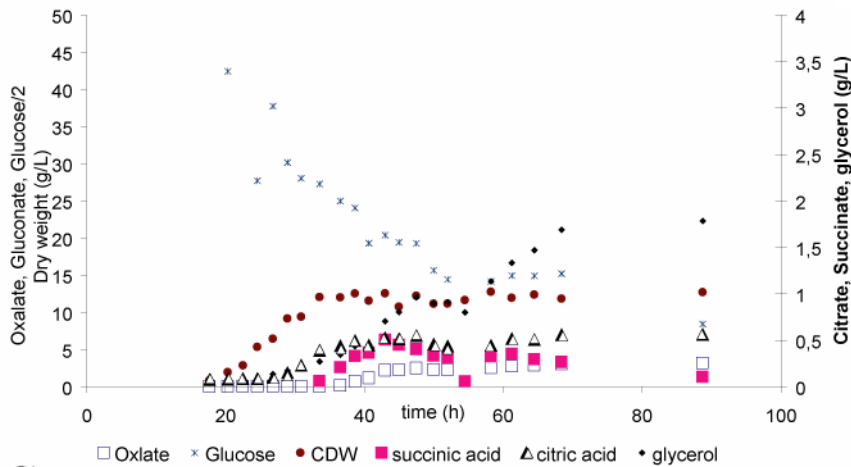
Figure 6.1 Initial strains characterization in pH 3.5 Peleg media (Peleg Y. *et al.*, 1988): A. N402 (WT), B. Frds(V), C. NW131(Δgox)

No improvement in succinate production was observed under these conditions for the *frds1* containing strain, but the Δgox strain did have a 2-fold increase in succinate over the WT. A delay in oxalate production, and increased glycerol production were observed for the Frds(V) strain. The major products for the WT and Frds(V) strains in the adjusted Peleg media were gluconic acid, while the Δgox strain produced no gluconic acid (as expected from its mutation) and up to 4g/L of oxalic acid.

Although the Frds(IX) strain was not tested in 5L-fermentations, it was employed further as it had the highest succinate yield in shake flask cultures. From continuous culture we found an increased intracellular succinate accumulation compared with the wild-type (a more detailed discussion of the continuous fermentations can be found in chapter 5). The extracellular succinate concentration was, however, below the detection limit of the HPLC, and it was therefore decided to insert a cytosolic fumarase to complete the pathway towards succinate production. Two transformants, a *Frds1* containing strain designated Frds(IX), and a transformant of Frds(IX) containing *Fum1s* (Frds(IX)Fum1s 1), and the wild type (N402) were fermented in well controlled 5-L fermenters at pH 3.5, using a medium designed for citrate production (Kristiansen B. and Sinclair C.G., 1979). No succinate was detected under these conditions (see Figure 6.2).
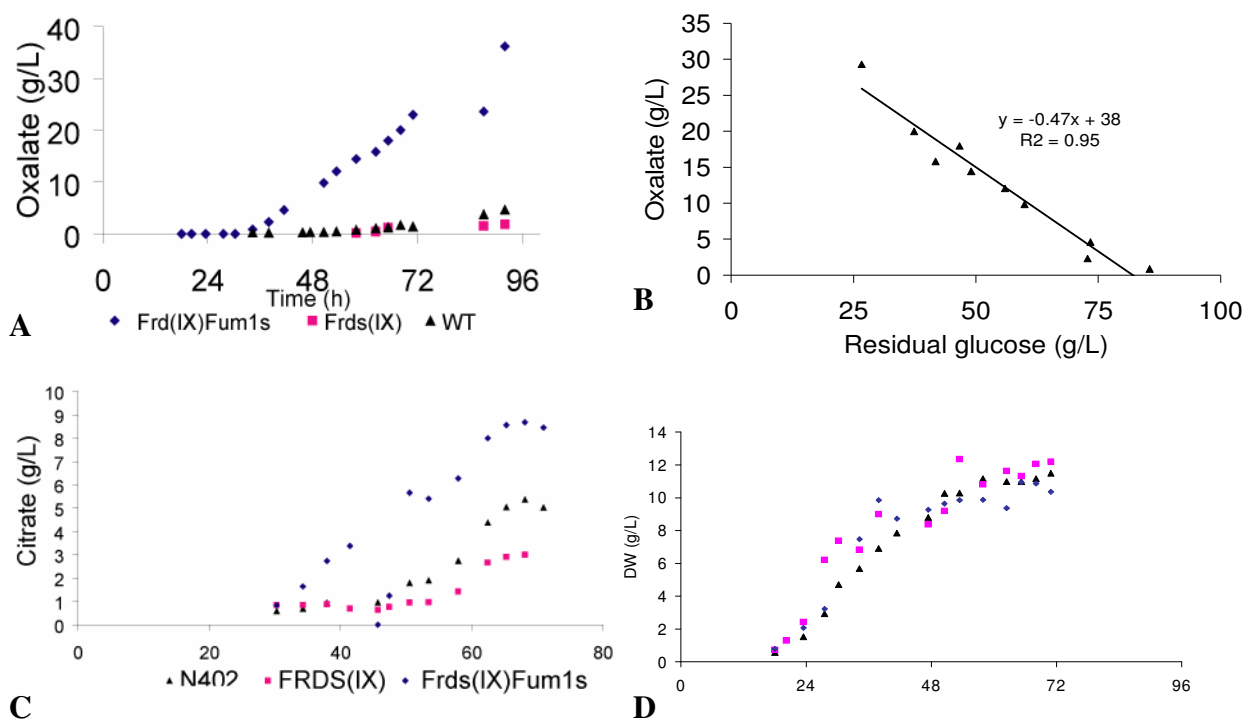
Figure 6.2. Fermentation at pH3.5 using Kristiansen media (Kristiansen B. and Sinclair C.G., 1979) with 100g/L glucose. A. Oxalate production for the three strains, B. Yield determined by plotting oxalate concentration vs. glucose concentration, C. Citrate production, D. Dry weight (DW).

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Frds(IX)Fum1s exhibits extremely high oxalate production under these conditions. It appears that the insertion of a cytosolic fumarase (*Fum1s* or *FumRs*) results in an excessive increase in oxalate production (see chapter 7 where similar results were observed for fumarase overexpressing strains). The significant overproduction of oxalate (yield of 0.4 g/ g glucose), although interesting in itself, was undesirable in the context of generating a succinate over-producing strain. The increased oxalate production could, however, indicate increased oxaloacetic acid levels in the cells, and hence insertion of the two genes has clearly resulted in modification of the fluxes in the reductive branch of the TCA cycle. The production of oxalate is reported (Ruijter G.J. *et al.*, 1999) to be inhibited at pH conditions lower than 3. It was therefore decided to change the pH control profile for the next set of batch fermentations (see Figure 6.3). The initial pH was still set to 2.5 and then increased in a step wise fashion to 3.5, but unlike the previous experiment, the pH was decreased in the mid-exponential growth phase to pH 2.2. The changed pH control strategy did decrease the production of oxalate, but it also severely decreases the production of citrate for all strains.

Once again citrate production is enhanced in the Frds(IX)Fum1s strains (two different Frds(IX)Fum1s strains were tested here to be sure the effect of the insertion is accurately represented). Interestingly, it appears that Frds(IX), under these conditions has a succinate production profile similar to that of the WT, and it appears to produce succinate at a slightly higher rate than the WT towards the end of the fermentation. The increased succinate production rate resulted in a slightly higher final succinate yield for Frds(IX) after 200 hours, compared to the WT and other transformants. A second *Frds1* transformant, Frds(V), was also tested under these conditions, and surprisingly, it happens to be the best citrate, and worst succinate producer of all the strains tested.
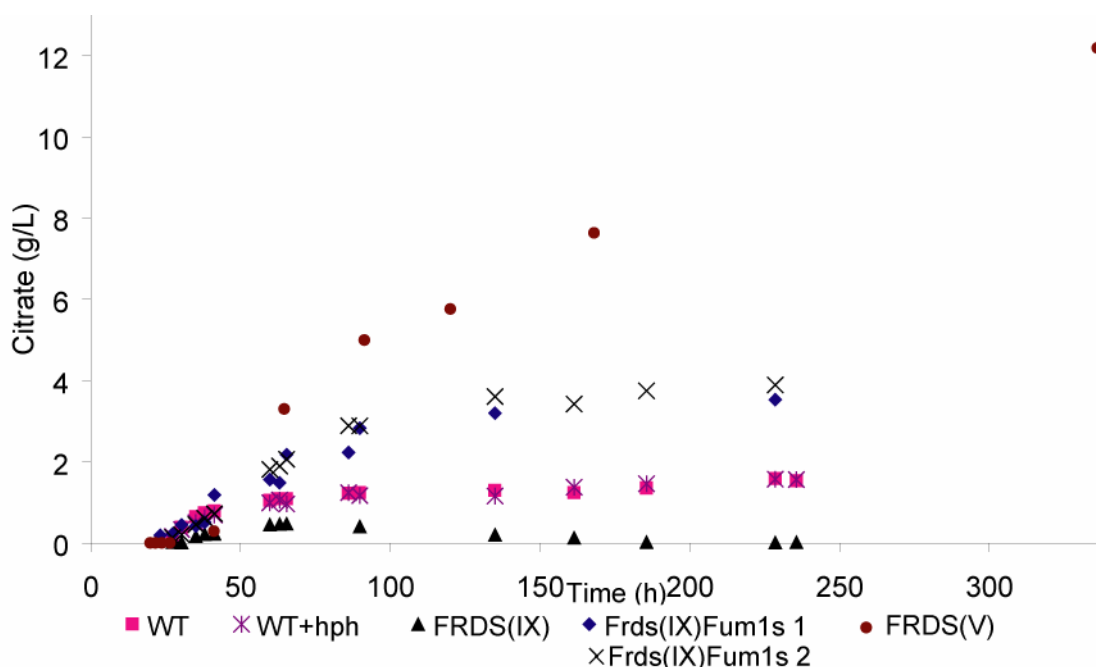
Figure 6.3A Citrate production in Kristiansen media with pH lowered mid-exponential to 2.2. The strain :Wt+hph, refers to a control strain of N402 transformed with an empty *hph* (hygromycin resistance marker) containing plasmid.
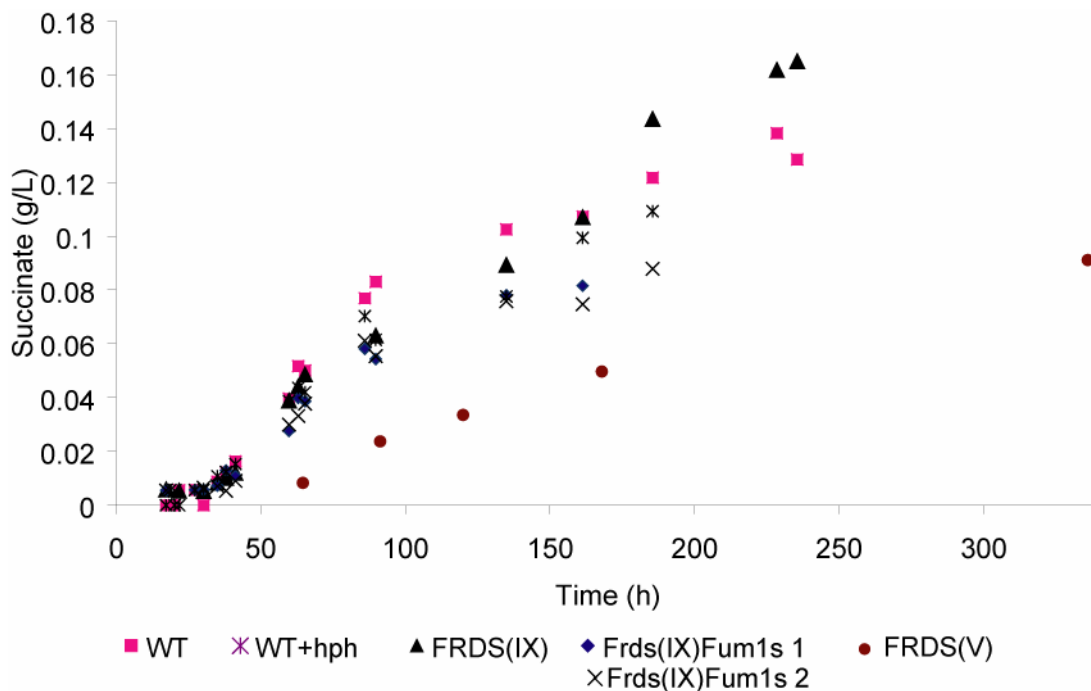
Figure 6.3B Succinate production in Kristiansen media with pH lowered mid-exponential to 2.2. The strain :Wt+hph, refers to a control strain of N402 transformed with an empty *hph* (hygromycin resistance marker) containing plasmid.

In a final attempt to increase succinate production the WT, Frds(IX)-Fum1s (overexpressing *Frds1* and *Fum1s*) and Frds(V)-FumRs1 (overexpressing *Frds1* and *FumRs,* see chapter 7) strains were cultured in conditions designed to result in oxygen limitation (see Figure 6.4 and Table 6.3). As was stated before, $FADH_2$ is extremely oxygen sensitive, and it was hoped that more of this co-factor would be available under oxygen limited condition, which would then allow for higher *frds1* activity. Also, the production of succinate occurs mainly through fumarate respiration by *frds1* in *S. cerevisiae* during anaerobic conditions (Arikawa *et al.*, 1998), and it was hoped that oxygen limiting conditions would adequately mimic anaerobiosis in *A.niger*.
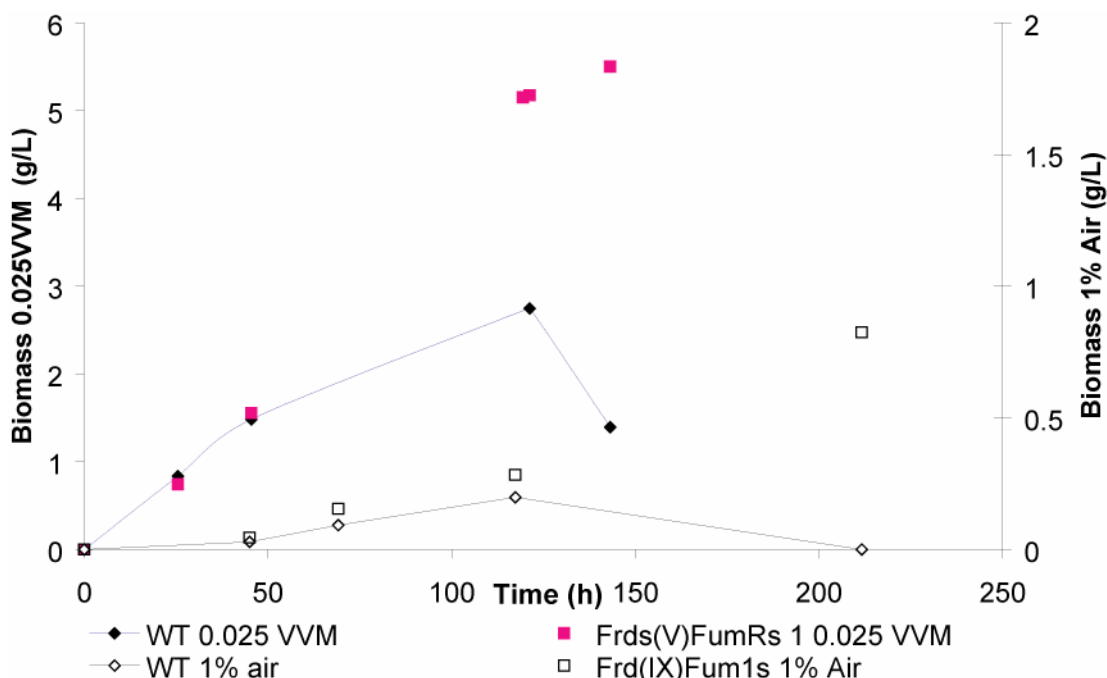
Figure 6.4. Oxygen limited growth and production profiles. The 1% Air fermentations were sparged with 1% air and 99%N$_2$ in Kristiansen media, pH 3.5, 50 g/L Glc, while the 0.025VVM fermentations were performed with air at 0.025VVM in Pedersen media (Pedersen H. *et al.*, 2000), adjusted to allow growth until 20 g/L biomass, pH 3.5, 100g/L Glc).

Table 6.3. Yields on biomass calculated at 120h for the 0.025VVM fermentation of the WT and Frds(V)-FumRs1.

Strain	Yxglycerol gGlycerol/gDw	YxEtOH gEtOH/gDw	YxMalate gMalate/gDw	YxFumarate gFumarate/gDw	YxSuccinate gSuccinate/gDw
N402 (WT)	0.35	0.37	0.17	0.01	n.d.
Frds(V)-FumRs	0.38	0.18	0.13	0.002	n.d.

The insertion of an alternative pathway towards succinate production in the cytosol results in enhanced growth under oxygen limitated cultivation conditions, compared to the wild type. Significantly higher biomass yields and production rates were achieved by the Frds(V)-FumRs 1 strain, and it was also able to achieve a much higher biomass concentration. The yield of glycerol on biomass was the same for the two strains, but the ethanol and fumarate yields were drastically reduced in the mutant. However, no succinate was produced by either of the two strains.

6.4 Discussion

The lack of significantly increased succinate production under the various conditions employed, or by the created strains, necessitated an in-depth analysis of the mechanisms underlying the production of the various observed products.

6.4.1 A lack of Zn^{2+} in adjusted Peleg et al medium leads to gluconic acid production

Initial characterization of the wt and *frds1* overexpressing strains was performed in a general organic acid production medium (modified from Peleg *et al.* 1988). This medium lacked Zn^{2+}, which results in a lowering of glycolytic capacity, as Aldolase is Zn^{2+} dependent (Mattey M., 1992). This in turn resulted in very high gluconic acid production rates, even though the pH was maintained at 3.5 (see Figure 6.1). This is far below the reported minimum gluconic acid production pH (Mattey M., 1992) In the model developed by David et al. (David H. *et al.*, 2003) for the central carbon metabolism it was however reported that there is also an intracellular pathway to gluconic acid production, which is therefore the most likely route to gluconate production under these conditions. The high gluconic acid production rate and yield, resulted in very little carbon being available for production of other organic acids. Succinate was only transiently produced to a maximum concentration of 0.25g/L for the WT and 0.2-0.25g/L for the *frds1* overexpressing strain. The Δgox strain, being unable to produce gluconate, produced oxalate, glycerol and a transient concentration of 0.5 g/L succinate, which was 2-fold higher than either the WT or Frds(V). The higher yield of succinate for the Δgox strain shows the importance of reducing by-product formation. The initiation of succinate production coincided with glycerol reconsumption for the WT, while this did not appear to be the case for the mutant strain. Succinate was also found to be reconsumed when glucose was exhausted, which could be potentially problematic for industrial applications.

6.4.2 Enhanced oxalic acid production by the double insertion mutant Frds(IX)-Fum1s

The problem of excessive gluconic acid production was resolved by changing to a new medium designed for citrate production (Kristiansen and Sinclair, Figure 6.2). The Kristiansen and Sinclair medium, unfortunately, resulted in the double insertion mutant (Frds(IX)-Fum1s) producing oxalate at rates greater than the values reported for strains and conditions designed for oxalate production (see Table 6.4). The production of oxalate is particularly problematic for the cytosolic fumarate respiration pathway, as *OAH* (oxaloacetate hydrolase) utilizes OAA (oxaloacetate) as substrate, which is also the substrate of malate dehydrogenase. This results in a redirection of carbon away from fumarate, the substrate for the inserted fumarase and finally fumarate reductase, towards oxalate, which is essentially a competitive by-product.

The insertion of *fumRs* did not lead to increased fumarate production. Friedberg et al (Friedberg *et al.*, 1995) found a direct link between *fumR* expression and the production of fumarate, although it was suggested that there were other effects involved. The

employed conditions, and specifically the activity of pyruvate carboxylase, appear to be the major contributing factors. As has been stated before, malate or fumarate production in *A. flavus* and *R. oryzae* only occurs in specially designed media, containing very high concentrations of CaCO3, which appears to lead to high pyruvate carboxylase activity.

The lack of fumarate production by the *fumRs* overexpressing strain also points towards pyruvate carboxylase being a limiting factor in the effective production of succinate through the inserted cytosolic pathway. Overexpression of PEPC, which has proven to be a very effective strategy in bacteria for increased succinate production (Lin *et al.*, 2005c), could therefore improve succinate production in the double overexpression strains. Using the Peleg malate production media (with 10% $CaCO_3$) could also lead to improved succinate production for the engineered strains, but this media has been shown to be extremely difficult to work with and would almost certainly lead to very high gluconic acid yields in *A. niger*. The high gluconic acid yields would be caused by the high pH (5-7) of the fumarate production media.

6.4.3 Is the inserted Frds1p enzyme active?

A question, which arises from the lack of succinate production in the *frds1* overexpressing strains, is: *Do we have any enzyme activity under aerobic conditions?* Unfortunately, no *in vitro* enzyme assay can easily answer this question, as the intracellular antagonistic effects of O_2 would be hard to mimic. Fortunately, the insertion of *frds1* results in a very specific metabolic footprint for all the tested overexpressing strains (including different transformants of *frds1*, namely Frds(IX) and Frds(V)). This metabolic footprint (perhaps tartaric acid), a specific peak detected by UV in HPLC, was shown to be unique and reproducible, and present even in the strains containing both *frds1* and a fumarase. The identity of this product will hopefully illuminate the role of *frds1* in its production, as it could be the increased availability of succinate, or the increased cytosolic availability of FAD^+, which leads to its production. A second strong indicator of *frds1* activity was demonstrated by the increased intracellular succinate concentration in continuous fermentation compared to the WT and a second mutant strain.

The drastic increase in citrate production was also another effect of inserting *frds1* (see Figure 6.3). The argument will be made in chapter 7 that the cytosolic produced succinate is imported by the mitochondrial citrate transporter into the mitochondria in exchange for citrate. This would explain the failure of the inserted cytosolic pathway to produce succinate extracellularly, even though the insertion of *frds1* has been shown to increase intracellular succinate concentrations (see chapter 5).

6.4.4 Comparison of succinate production in two *frds1* containing transformants indicates varying roles of *frds1*

If we compare the production of succinate and citrate between two *frds1* overexpressing transformants, Frds(IX) and Frds(V), we see how increased succinate production coincides with decreased citrate production, and vice versa. It appears that if the

intracellular conditions of the strain are conducive to citrate production, less succinate is produced. It is hard to conclusively explain the difference between these two transformants. Both display the above mentioned metabolite footprint, indicating *frds1* activity. The level of activity is not known, however, and it could be argued that the difference between the strains was depending on the expression level of *frds1*. It has been mentioned before that *frds1* is involved in maintaining the intracellular NADH/ NAD$^+$ redox balance under anaerobic conditions in yeast.

The production of citrate has a similarly unbalancing effect on intracellular redox as anaerobiosis in yeast, both of which leads to excess NADH production. Therefore citrate production normally requires very specific fermentation conditions. It is currently theorized that these conditions lead to high ATP consumption by active export of citrate from the cell at low pH, and concurrently the action of the alternative oxidase to convert NADH to NAD$^+$, without concomitant ATP formation, is very important. A high enzymatic activity of *frds1* could therefore facilitate citrate production, by not only providing a cytosolic alternative to malate for citrate antiport from the mitochondria, but also through maintaining the NADH/ NAD$^+$ redox balance. A low level of frds1p activity would therefore lead to less intracellular succinate being formed, compare to the highly overexpressed *frds1* strain, but it would not lead to citrate production, as it cannot maintain the required conditions intracellularly. The produced succinate would therefore be exported, instead of it being consumed, as is the case for the higher frds1p activity strain.

Another theory could be that the random insertion of *frds1* in Frds(IX) led to a disruption event, which, while not appearing to drastically affect strain growth or general metabolite production profiles, negatively influences citrate production. The lack of citrate production then directly improves succinate production, as succinate is not imported into the mitochondria in exchange for citrate any longer. However, there are results that argue against a pleiotropic disruption event during transformation. Firstly, detailed comparison of the WT to Frds(IX) revealed only very minor differences between the strains in chemostat experiments, and secondly, the insertion of *fum1s* into Frds(IX) led to drastically enhanced citrate production. A more detailed study on *frds1* expression level and intracellular succinate concentrations between Frds(IX) and Frds(V) would be needed to differentiate between and prove these theories.

6.4.5 Effect of alternative cytosolic pathway insertion on strain physiology under oxygen limited fermentation conditions.

Oxygen limited fermentations with the WT and double insertion mutants, containing fumarase and fumarate reductase, revealed a higher growth rate under these conditions. It was also found that the WT starts to grow on the walls and internal parts, which can be observed as a decrease in the biomass concentration in Figure 6.4. The wall growth probably indicates a stress response to oxygen starvation. It therefore appears that the transformants are better able to grow under oxygen starved conditions. This ability can be explained by the oxidation of NADH to NAD$^+$ through the action of frds1p. The direct mechanism by which Frds1p was able to do this is not understood at this time, but

a similar observation was made in yeast (Enomoto *et al.*, 2002). Another clear indication of the redox-influencing role of frds1p was the two-fold decrease in ethanol production compared to the WT. It is surprising that no succinate is produced, as the enzyme is clearly active, as can be deduced from the decreased ethanol production. Yeast produces succinate mainly through this enzyme under similar conditions. It therefore appears that *A. niger* has the capacity to metabolize the produced succinate before it can be excreted. The mechanism of succinate metabolism under these conditions could involve import into the mitochondria, or use as a precursor during growth.

The high rate of EtOH production by the WT under oxygen limiting conditions supports the prediction by David et al. (David H. *et al.*, 2003) that the deletion of pyruvate decarboxylase could enhance succinate production under oxygen limiting conditions. Gallmetzer et al. (Gallmetzer *et al.* 2002) suggested that fumarate respiration helped maintain the redox balance in *P. simplicissimum* in oxygen limited conditions, and was responsible for the observed increase in succinate production. It should also be stated that, unlike *A. niger* strain N402, *P. simplicissimum* does not produce ethanol in oxygen limited conditions.

6.5 Conclusion

In summary, in an effort to produce succinate, we managed to produce several organic acids, often at values equaling or even surpassing published yields and productivities (see table 6.4). A theory towards explaining the mechanism preventing succinate production has also been proposed.

Table 6.4 Comparison of achieved and previously reported organic acid production rates and yields

	Ysp (g / g glc)	rp(g/L.h^{-1})	Titer (g/L)	Values in Literature
Oxalate	0.4 (0.65)	0.4$_{total}$ / 0.6$_{max}$ (0.29)	36 (38g/L in fed-batch)	(Strasser *et al.*, 1994); (Van de Merbel N. *et al.*, 1994)
Citrate	0.46$_{total}$ / 0.9$_{max}$ (>0.8)	0.19$_{max}$ (0.7-1)	20 (>120)	(Mattey M., 1992)

Values between brackets represent values reported in literature for *A. niger* by the authors listed in the final column.

$_{Total}$ indicates the yield or productivity calculated from the final sample point of he fermentation
$_{max}$ indicates the value corresponding to the maximum sustained yield or productivity measured during the batch fermentation

The production of large quantities of by-products was reduced by media optimization. However, the very changes that negatively affect by-product formation could lead to lower succinate productivity by lowering the efficiency of the inserted cytosolic pathway. This is especially true for oxalate production, as oxalate is produced from OAA, which is a precursor for the inserted pathway. The deletion of OAH is therefore a very important

target for future improvements to succinate production using the inserted alternative cytosolic pathway strains. The importance of using gene deletion instead of media optimization was also demonstrated with the Δgox strain, which was able to produce 2 fold more succinate in the modified Peleg media, while media optimization to prevent gluconic acid production in the WT led to lower succinate production.

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Chapter 7. Enhanced citrate production through gene-insertion in *A. niger*

7.1 <u>**ABSTRACT**</u>

The effect of inserting genes involved in the reductive branch of the TCA cycle on citrate production by *A. niger* was evaluated. Several different genes were inserted individually and in combination, i.e. malate dehydrogenase (*mdh2*) from *S. cerevisiae*, two truncated, cytosolic targeted, fumarases (*Fum1s* and *FumRs*) from *S. cerevisiae* and *R. oryzae*, respectively, and the cytosolic soluble fumarate reductase (*Frds1*) from *S. cerevisiae*. Over-expression of these genes in their native strain backgrounds have been reported to lead to alterations in the intracellular cytosolic dicarboxylate concentrations. It was found that all the transformant strains had enhanced yield and productivities of citrate compared with the wild-type strain. The transformants also had the ability to produce citrate in trace manganese contaminated medium, where the wild-type was unable to produce. The best citrate producing strain in trace manganese contaminated conditions, had overexpression of both *FumRs* and *Frds1,* and this strain had a maximum yield of 0.9 g citrate per g glucose and a maximum specific productivity of 0.025g citrate per gDW per hour. Over-expression of *mdh2* alone resulted in an increased citrate production rate in the initial phase of the fermentations compared with the other transformants and the wild-type.

7.2 <u>Introduction</u>

Citrate is the most intensively produced fungal bulk chemical product, with a global market of more than 900 000 tons/ year (2001). *Aspergillus niger* fermentations meets the biggest part of this demand. Yet, despite decades of study, only a small part of the production process is understood in detail (Karaffa L. and Kubicek C.P., 2003). It has long been suspected that the principal mode of citrate export from the mitochondria involves the anti-port of cytosolic malate by the mitochondrial citrate transporter (CTP) (Ruijter G.J.G., Kubicek C.P., and Visser J., 2002). Specificly, Kubicek et al. (Rörh and Kubicek, 1981) found convincing evidence for an increase in cytosolic malate directly preceding the onset of citrate accumulation.

Mathematical modelling has been extensively employed in the study of citrate production. Using a metabolic flux balance model it was predicted that one of the rate limiting steps is citrate export from the mitochondria (Guebel D.V. and Torres Darias N.V., 2001). Unfortunately the mitochondrial citrate transporter (CTP) has only been identified in *S. cerevisiae*, and although some sequence similarity was found at the protein level (40% identity and 55% similarity, with an e-value of 4E-50), it has not yet been cloned in *A. niger*. Interestingly, in *S. cerevisiae*, a connection has been demonstrated between cytosolic malate accumulation and extra-cellular citrate production (Pines *et al.*, 1996). The overexpression of the cytosolic enzymes, MHD2p and FUM1p, both results in increased malate, as well as increased citrate production in yeast (Pines *et al.*, 1997).

No detailed study has so far been reported on the affinity of CTP to different dicarboxylic acids in *A. niger*, but it has been reported that the transport of citrate across the mitochondrial membrane is influenced by malate, isocitrate, succinate and phosphoenolpyruvate in *S. cerevisiae* (Sandor *et al.*, 1994). This result can be supported by employing 3D-structural prediction using CPHmodels2, followed by 3D-structural comparison using (http://cl.sdsc.edu/), which showed that there were great similarities (Rmsd = 1.1Å Z-Score = 6.3) between the *S. cerevisiae* DIC1p and CTP1p. The 3D structures overlap very well for approximately half (161 aminoacids) of the CTP1p (282 aminoacids) and DIC1p (298 aminoacids) proteins, indicating that it is probably the cytosolic facing part of the CTP1 transporter, which has very high similarity to the DIC1 protein. The possibility that the affinity of CTP for malate, succinate and other organic acids, at least in some aspects, mimics the affinity of the DIC1 transporter, poses the intriguing question: Can mitochondrial citrate export be increased by increasing the cytosolic concentration of different 4-carbon dicarboxylic acids in *A. niger*.

The tight regulation of the central carbon metabolism of *A. niger* have been amply demonstrated by the lack of success in directly increasing citrate productivity through over-expression of central carbon metabolism genes thus far. Torres and co-workers used biological systems theory coupled with constrained linear optimization, to show that at least 7 glycolytic enzymes needed to be over-expressed to achieve a significant increase

in flux towards citrate (Torres N.V. *et al.*, 1996). This helps to explain the disappointing results obtained by the single (citrate synthase (Ruijter *et al.*, 2000)) and double (pyruvate kinase and phosphofructokinase (Ruijter *et al.*, 1997)) over expression of genes involved in citrate biosynthesis from glucose. The only successful attempts have been through indirect means: disruption of the trehalose-6-phosphate synthase A (T6PSA)-encoding gene (ggsA), which leads to decreased inhibition of hexokinase by trehalose-6-phosphate (Arisan-Atac *et al.*, 1996); and anti-sense expression of *Brsa-25* (a possible amino acid transporter), which allows the formation of citrate-production-enhancing pelleted growth in the presence of normally inhibiting Mn^{2+} concentrations.

The compelling results in *S. cerevisiae*, coupled to the probable similarities in dicarboxylate affinity between CTP and DIC, therefore led us to investigate the impact of altered cytosolic organic acid concentrations as an indirect approach towards studying and enhancing citrate productivity in *A. niger*.

7.3 <u>Experimental procedures</u>

Fungal strains. All Aspergillus niger strains used in this study were generated from the A742 (cspA1 pyrA5) reference strain.

7.3.1 Plasmids. Manipulation of plasmid DNA and transformation of plasmids into *Escherichia coli* DH5α were carried out according to standard procedures (Sambrook, J. and Russell, D. W., (2001)). All plasmids were constructed as random genomic integrating vectors. Three of the genes (*Fum1*, *FumR*, and *Mdh2*) used in this study were over-expressed by introduction of these genes into the pAT-1 vector (see table 2).

MDH2, Fum1s and *FumRs* were separately introduced into plasmid pAT-1 by ligating, respectively, a 1.3kb, 1.4kb and 2 kb PCR product of *MDH2* from *S. cerevisiae* (using primer pair: Mdh2_F and Mdh2_R, see primers in table 1)*,* a truncated *Fum1* from *S. cerevisiae* (using primer pair: Fum1s_F and Fum1s_R) and a truncated *FumR* from *R. oryzae,* (using primer pair: FumRs_F and FumRs_R) into pAT-1. The truncated version of *fum1* and *fumR* will be referred to as *Fum1s* and *FumRs* in this work. The PCR primers were designed to contain a BsaI cut site on the 3' primers and a NotI cut site on the 5' primers. The PCR products were then digested with the offset cutting BSAI (designed to result in a 5-prime overhang matching to the 3-prime overhang of NcoI in pAT-1) and NotI, into pAT-1 cut with NcoI and NotI. The truncated version of *Fum1* and *FumR* (referred to as *Fum1s* and *FumRs*) were designed to lack the initial 17 and 15 amino acids respectively. The designation of the different plasmids used in this study can be seen in table 2.

The final gene used in this study, *FRDS1*, was placed on a plasmid through several steps:
1. BamHI-HindIII fragment, containing the terminator region from *A.nidulans trpC* gene, was excised from pAN7-1, and inserted into BamHI and HindIII sites of pUC57 (Fermentas), to construct plasmid pUC57Ter-trpC.
2. Promoter region of the *A. nidulans gpdA* gene from plasmid pAN7-1 was amplified by PCR with primers PAN1 (5'-gtg-agt-gga-tcc-ata-tgg-atg-tct-gct-caa-gcg -3',

containing NdeI and BamHI sites) and M13/pUC reverse sequencing primer (Fermentas). Resulted DNA-fragment was digested with EcoRI and BamHI, and cloned into EcoRI and BamHI sites of pUC57TtrpC to create the plasmid pAN-N.

3. NdeI site was disrupt in ppyrG (Oakley *et al.*, 1987), by step by step digestion with NdeI, treatment with Klenow fragment DNA polymerase I and dNTP, and ligation by T4 DNA ligase. Resulted plasmid was designated as ppyrG-N.

4. SspI-EcoRI fragment, contained *pyrG* and disrupted NdeI site, was excised from ppyrG-N, and inserted into SspI and EcoRI sites, to construct plasmid pUC57Ter-trpC-pyrG-N.

5. pUC57Ter-trpC-pyrGN was consequently treated with XbaI and T4 DNA ligase, to delete XbaI-XbaI fragment. Resulted plasmid was step by step treated with BamHI; Klenow fragment DNA polymerase I and dNTP; and T4 DNA ligase, to disrupt BamHI site in pyrG.

6. EcoRI-XbaI fragment, contained promoter region of the A. *nidulans gpdA* and terminator region from *A.nidulans trpC*, was excised from pAN-N and cloned into pTer-trpC-pyrG-NB-Xba, to create pANNPYRG.

Yeast *frds1* was amplified by PCR with primers: frds13 (5'-aat-tgc-ata-tgt-ctc-tct-ctc-ccg-3, contained NdeI site) and frds12 (5'-tat-agg-atc-cgt-tac-ttg-cgg-tca-3', contained BamHI site). Resulted DNA-fragment was digested with NdeI and BamHI, and cloned into NdeI and BamHI sites of pANNPYRG to create the plasmid pFRDS-A1.[1]

Table 7.1 Sequences of primers used to create pFum1s, pFumRs and pMDH2

Primer name	Sequence
Fum1s_F	gtctaGGTCTCCCATG**AACTCCTCGTTCAGAACTGAAAC**
Fum1s_R	GCGGCCGC-**TTATTTAGGACCTAGCATGTGTTCAGG**
FumRs_F	gtctaGGTCTCCCATG**AACAACTCTCCTCGTCTTTTCAG**
FumRs_R	GCGGCCGC**TTTAATCCTTGGCAGAGATCATATCTTC**
Mdh2_F	gtctaGGTCTCCCATGATTCTCCTTCTGATTCTTTTTCCC
Mdh2_R	TAGCGGCCGCTTAAGATGATGCAGATCTCGATGC

Table 7.2 Main plasmids used in this study

Plasmid name	Created	Description
pFum1_s	This study	Contains *S. cerevisiae Fum1s* expression cassette
pFumR_s	This study	Contains *R. oryzae FumRs* expression cassette
pFrds1_A1	This study	Contains *S. cerevisiae Frds1* expression cassette
pMdh2	This study	Contains *S. cerevisiae Mdh2* expression cassette
pAN7-1	(Punt *et al.*, 1987)	Contains *E. coli* h*ph* gene, confers hygromycin resistance.
pAT-1	(Tolsters A., 2003)	Contains strong constitutive fungal gpdA promoter and trpC terminator.
pGemT-*pyr4*	Ligation of PCR product into an Invitrogen pGemT plasmid	Contains *N. crassa pyr4* gene, cures a *pyrG* mutation (piramidine

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[1] The authors gratefully acknowledge A. Aleksenko for the creation, including all the intermediate cloning steps, of pFRDS_A1.

7.3.2 Fungal Transformation. PyrG- mutant A742 was transformed with plasmid pFRDS A1 containing the *Frds1* gene and *A. niger pyrG* as marker to create strain Frds(V). These strains were then co-transformed with pFum1_s or pFumR_s, and pAN7-1 to create strains Frds(V)-Fum1s and Frds(V)-FumRs. A control strain was created where N402 was transformed with pAN7-1 alone, it will be referred to as Control in the text. *PyrG-* mutant A742, which has the same strain background as wild type *A. niger* **N402**, were co-transformed with pFum1s, or pFumRs, or pMdh2 and plasmid pGemT-pyr4 containing *N. crassa pyr4* as a marker gene. The co-transformation ratio was 10:1 to the marker plasmid. Transformation of *A. niger* was achieved as described by (Michael L.Nielsen *et al.*, 2005) for *A. nidulans*. 11µl of plasmid were added to a 100µl volume containing at least 10^6 protoplasts and plated in selective media. The resulting strains are designated **Fum1s**, **FumRs** or **MDH2**, respectively.

7.3.3 Media for strain development. *A. niger* strains were grown at 30°C on solid or liquid minimal media (MM) (Clutterbuck, A. J., (1974)) or MM containing uracil and uridine for *pyr*- auxothrophic strains. Selection of transformants were performed either on MM media or MM containing 200-500 µg/ml hygromycin B. Bacterial strains were grown at 37°C in LB medium with 100 µg ampicillin/mL.

7.3.4 Inoculum. The strains were stored at -80°C in eppendorf tubes containing 0.01% Tween with 20% (v/v) glycerol. From the stock tubes, point inoculations with a toothpick on plates with selective media for plasmid bearing strains were used.

7.3.5 Cultures grown in shake flasks. Baffled, cotton-stopped, 500 mL Erlenmeyer flasks were used. These flasks contained a volume of 100 mL medium with the following compositions: MM media when protoplasts for fungal transformations were to be obtained, or Kristiansen media (Kristiansen B. and Sinclair C.G., 1979) for screening of transformants. The pH was adjusted to 5.2 with NaOH and autoclaved along with the glucose solution. The shake flasks were grown at 30°C and 150 rpm for up to 2 weeks.

7.3.6 Medium for the batch cultivations. The medium for the batch cultivations had the following composition: 50g/L NH_4NO_3; 50g/L KH_2PO_4; 10 g/L $MgSO_4 \cdot 7H_2O$; 1 mL/L 1000 X trace element solution containing per litre: 0.037g/L $FeCl_3$, 0.06g/L $CuSO_4.5H_2O$, 0.1g/L $ZnSO_4.7H_2O$; 50 µL/L antifoam (Sigma, A-8436); and 50 g/L glucose. (Kristiansen B. and Sinclair C.G., 1979)

7.3.7 Cultivation conditions. The cells were grown as aerobic batch cultivations with nitrogen eventually being the growth-limiting component. The cultivations were carried out in well-controlled 5-litre in-house-manufactured bioreactors with working volumes of 4 litres. The bioreactors were equipped with two disk turbine impellers rotating at 500 rpm. The pH was initially set to 5, 150 rpm agitation, and with an airflow of 0.1 VVM. After 16 hours the airflow was then increased to 4 litre per min. (1 VVM), and the agitation set to 500rpm. The pH was allowed to decrease without further control. The off gas passed through a cooled condenser to limit evaporation from the bioreactor.

7.3.8 Cell mass determination. The cell mass concentration on a dry weight basis was determined by the use of nitrocellulose filters with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, Mich.). Initially, the filters were pre-dried in a microwave oven at 150 W for 10 min., and then weighed. A known weight of cell culture was filtered, and the residue was washed with distilled water. Finally, the filter was dried in the microwave at 150 W for 15 min., and then weighed.

7.3.9 Analysis of extracellular metabolites. For determination of the extracellular metabolites, a sample was taken out of the bioreactor and immediately filtered through a 0.45 μm-pore-size cellulose acetate filter (Osmonics, Minnetonka, MN). The filtrate was frozen and kept at -20°C until analysis. Organic acids and glucose were separated on an Aminex HPX-87H column (Biorad, Hercules, Calif.) at 65°C, using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and detected refractometrically (Waters 410 Differential Refractometer Detector; Millipore Corp., Milford, Mass.).

7.4 <u>Results</u>

The suggested role of the mitochondrial citrate antiporter was tested through the insertion of several genes (*MDH2, Frds1, FumR, Fum1*) into an *A. niger* wild-type and characterizing the mutants in a citrate production medium. The genes were selected for insertion based on their published ability, be it in different organisms (*S. cerevisae* and *R. oryzae*), to affect the accumulation of different organic acids in the cytosol [(Muratsubaki and Katsume, 1985);(Friedberg *et al.*, 1995);(Peleg *et al.*, 1990);(Pines *et al.*, 1997); (Pines *et al.*, 1996)]. A summary of the fermentations performed in this study can be seen in figure 1.
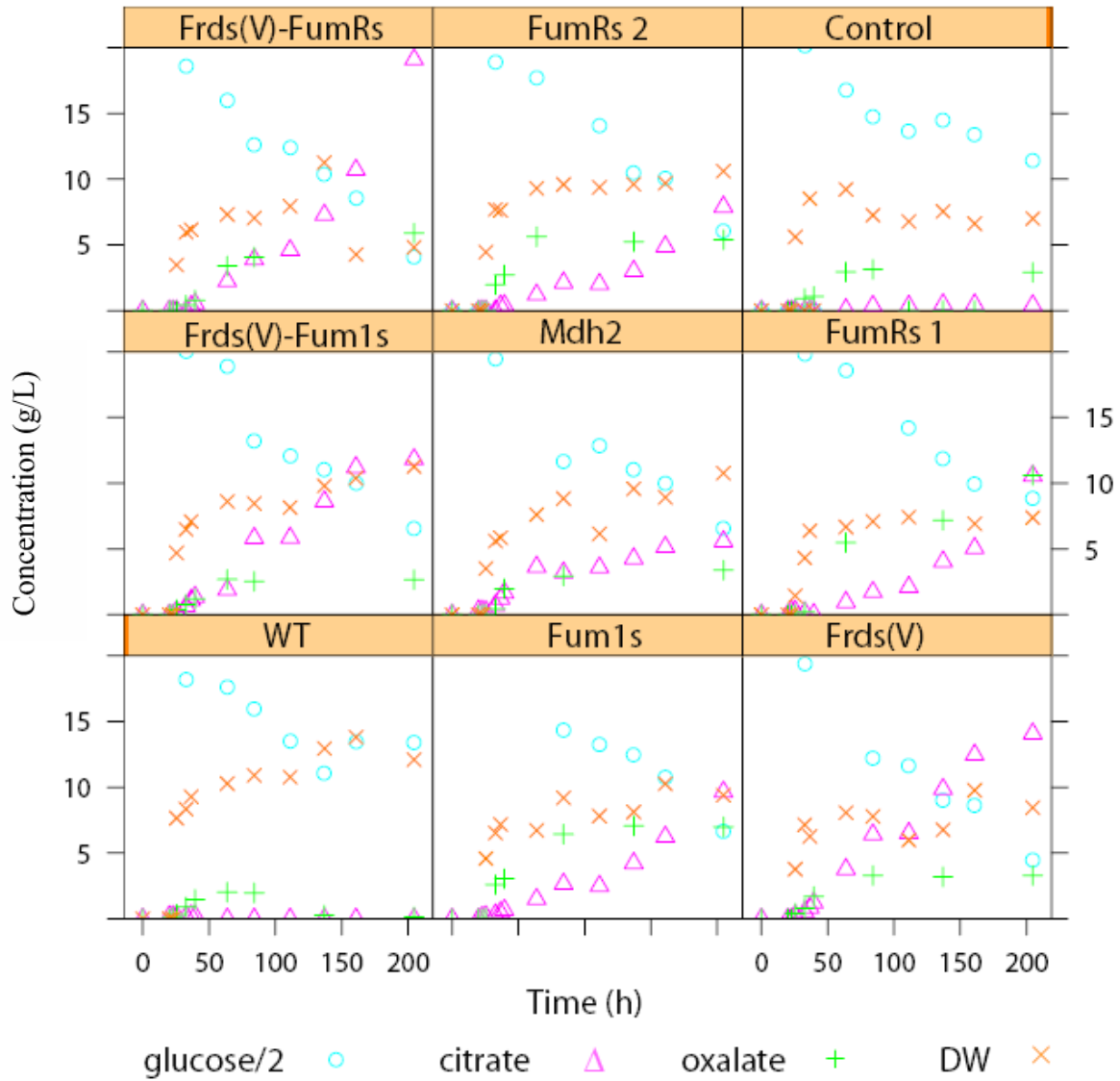
Figure 7.1. The major fermentation products, as well as glucose concentration (plotted as the measured value divided by 2), were plotted for all the attempted fermentations. The strain name is printed at the top of each panel.
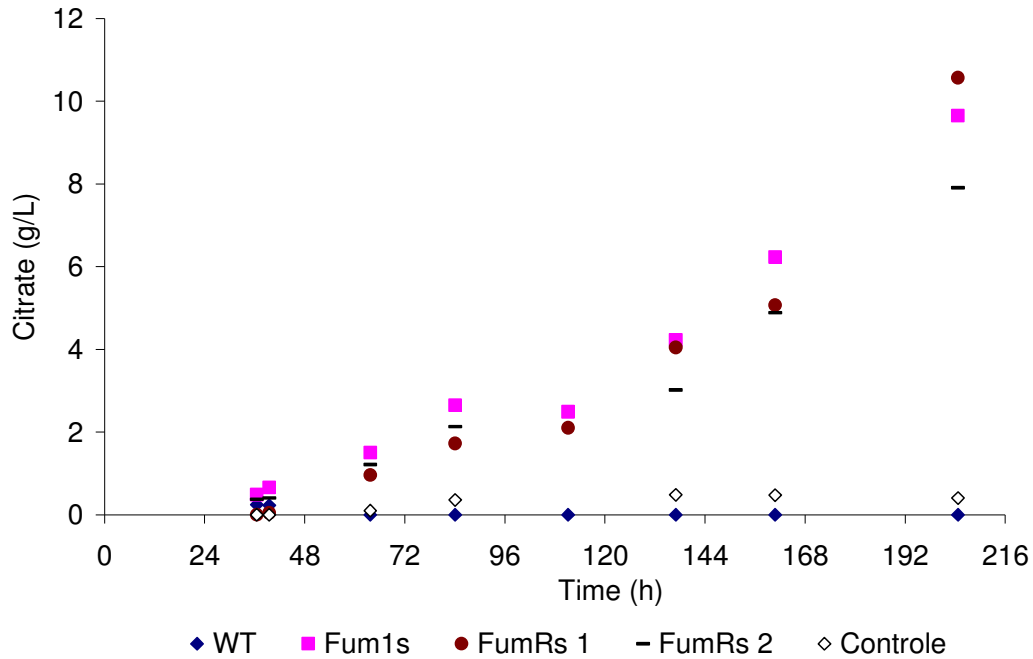
Figure 7.2. Effect of cytosolic fumarase overexpression on citrate production.

Significantly increased citrate productivities and yields were observed when two different cytosolic targeted fumarases, *fum1* and *fumR* (from *S. cerevisiae* and *R. oryzae,* respectively), were expressed. The strains were compared under several different conditions, including shake flask cultures and different batch cultivation conditions (results not shown). In all instances higher yields and productivities were observed for the strains containing *fum1s* or *fumRs,* compared with the wild type. From figure 2 it can be seen that the wild type and control strains do not produce citrate in the batch fermentations, however, the wild type did produce citrate in shake flask culture, but still at a slower rate than the transformants.
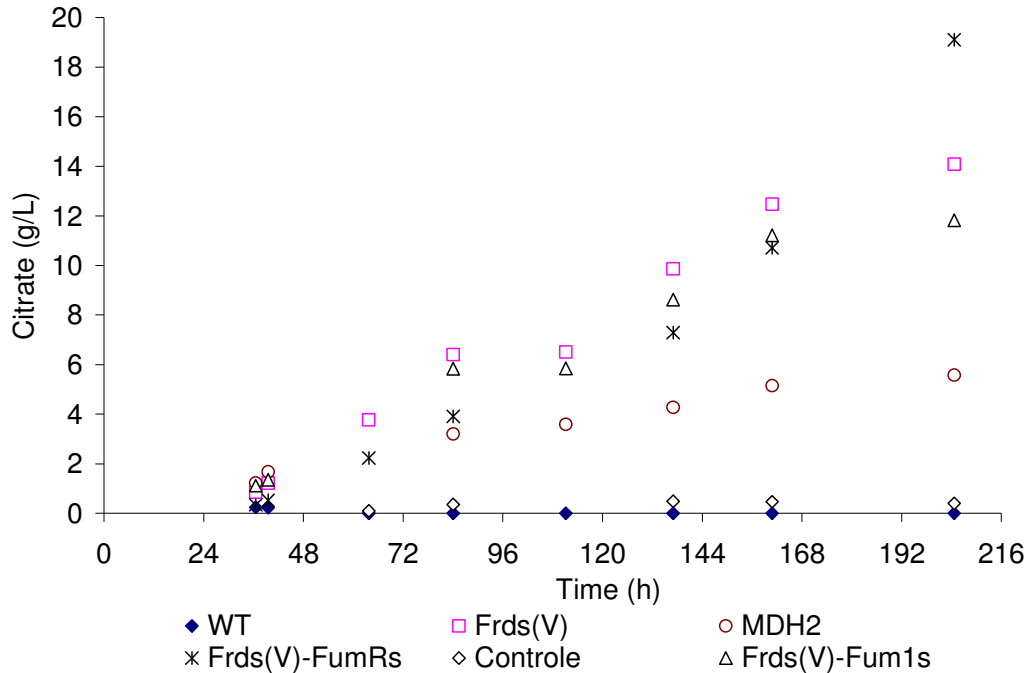
Figure 7.3. Effect of the insertion of *Frds1* and *MDH2,* as well as the effect of the secondary insertion of *fumRs* or *fum1s* into the *frds1* containing Frds(V) strain.

In figure 3 the effect of expressing *S. cerevisiae Frds1* can be seen. Once again higher citrate yields and productivities were obtained. Indeed, the *frds1* containing transformants had the highest productivities of all the strains tested. The insertion of *fumRs*, but not *fum1s*, into the Frds(V) strain background, however, resulted in a further increased citrate production compared to the single gene insertion *frds1* (Frds(V)) strain, particular in the later stages of the fermentation.

Expression of the *MDH2* gene from *S. cerevisiae* also resulted in an increased citrate production compared with the wild type and control. When compared with the other transformants, it also results in an increase in the initial citrate production rate. However, except for the initial stages of the fermentation, it performs poorly compared to the other gene insertion transformants.
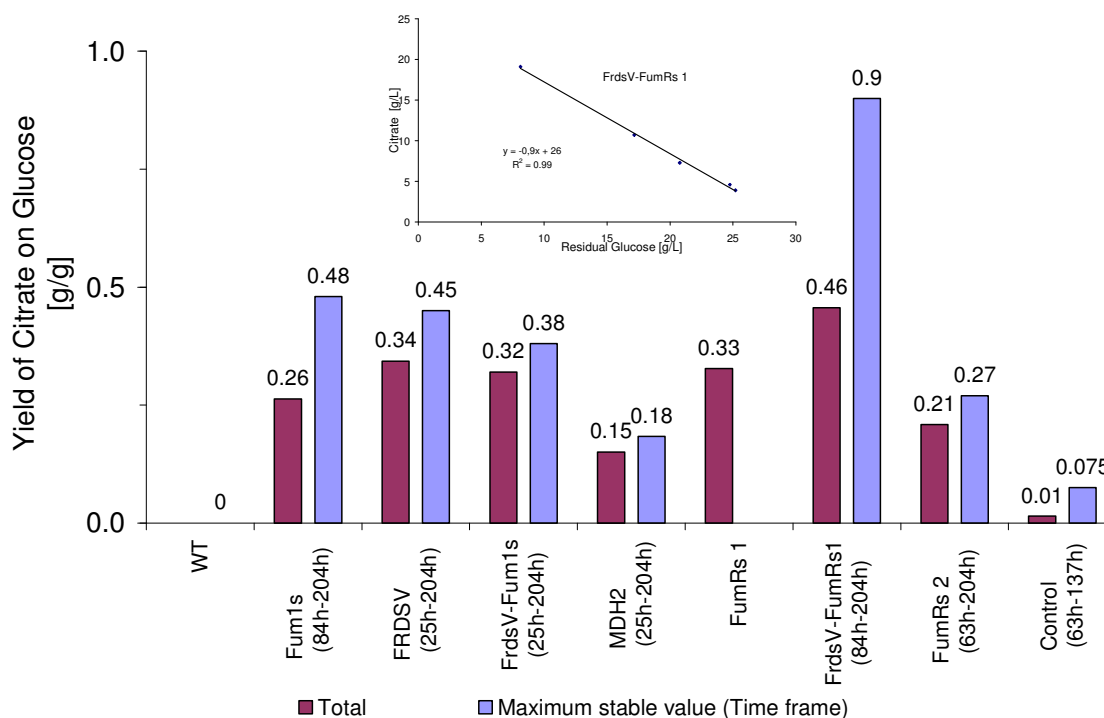
Figure 7.4. The citrate yield of all the tested strains are given as g citrate produced/ g glucose consumed. The total citrate yield represents the final citrate concentration divided by the consumed glucose. The maximum stable value represents the maximum sustained yield achieved during the fermentation, and the time frame in which it was achieved is given between brackets. The total fermentation time would be 0h-204h. As the yield of citrate on glucose continued to increase though out the fermentation for FumRs 1, no maximum stable yield could be determined. The inserted figure illustrates the citrate concentration versus residual glucose concentration in the time frame 84h to 204h for the Frds(V)-FumRs 1 strain, i.e. the data used to calculate the maximum stable value of the yield.

The yield of citrate on glucose, which is another important parameter for optimization of citrate production, is most significantly increased by the insertion of either *Fum1s* or *FrdsV,* or the dual insertion of *Frds1* and *FumRs* (see figure 4). A direct correlation between glucose consumption rate and citrate production rate was also found for the transformants, except for FrdsV-FumRs 1, which had a significantly higher citrate production rate compared to its glucose consumption rate. A very strong Pearsons correlation (higher than 0.98) for all the transformants were found between citrate and pyruvate production. A strong correlation between fumarate consumption rate and citrate production rate was also observed for al the transformants containing either a fumarase or fumarate reductase gene (see figure 5).
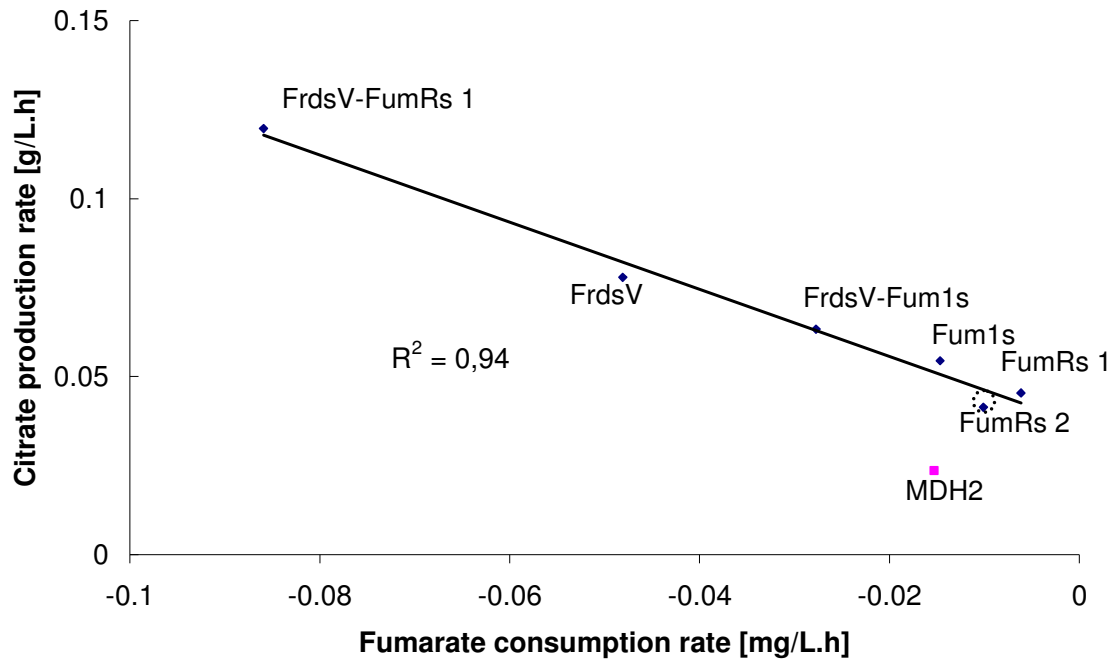
Figure 7.5. Citrate production rate versus fumarate consumption rate in the later stages of fermentation. The FumR2 point represents the citrate production rate calculated after excluding the final measurement, as the citrate production rate drastically increased for the final measurement and therefore does not represent the trend observed for the rest of the measurements.
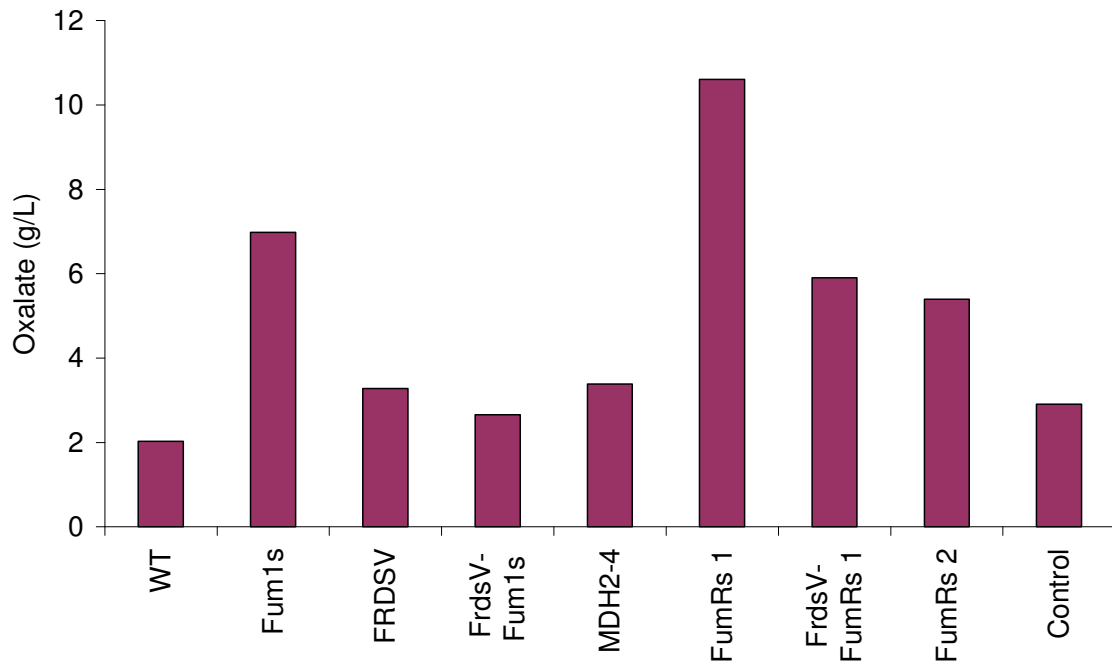
Figure 7.6. Effect of different gene insertions on final oxalate titer

The insertion of a cytosolic fumarase, be it *fum1* or *fumR*, results in a drastic increase in oxalate production and yield (see figure 6). The effect of *fumRs* insertion on oxalate

production was also evident when a fumarate reductase overexpressing strain was used as host strain for the transformation, although to a lesser extent compared to the insertion of *FumRs* into the wild-type.

7.5 **Discussion**

The aim of this work was to gain further insight into the mechanisms governing citrate over-production, and particularly the mechanisms behind citrate export from the mitochondria. By using a kinetic model for citrate production Guebel and Torres predicted that citrate export from the mitochondria is likely to be a rate limiting step for citrate production (Guebel D.V. and Torres Darias N.V., 2001). The validity of this prediction and the importance the citrate transporter in the onset of citrate production was therefore tested by characterising several mutants designed to lead to altered cytosolic 4-carbon dicarboxylate concentrations.

The truncated version of *Fum1* and *FumR* (referred to as *Fum1s* and *FumRs*) were designed to lack the initial 17 and 15 amino acids respectively, which, when removed in *S. cerevisae* ((Peleg *et al.*, 1990), (Wu and Tzagoloff, 1987)) and *R. oryzae* (Friedberg *et al.*, 1995), prevents the mitochondrial import of Fum1p and FumRp, and has been demonstrated to result in purely cytosolic activity for Fum1p.

Frds1 has been demonstrated to irreversibly convert cytosolic fumarate to succinate, using $FADH_2$ as co-factor. The expression of this gene in *A. niger* was therefore expected to increase the cytosolic succinate concentration. A secondary effect in the redox balance of the strain was also expected, as *frds1* has been shown to be essential for reoxidizing NADH to NAD^+ under anaerobic conditions in yeast (Enomoto *et al.*, 2002). Excessive NADH production is also a problem under citrate production conditions, which usually requires high alternative oxidase activity (AOX) to maintain the redox balance and limit the amount of ATP produced (Ruijter G.J.G., Kubicek C.P., and Visser J., 2002).

7.5.1 Initiation of citrate production

Rörh and Kubicek (Rörh and Kubicek, 1981) observed intracellular malate accumulation directly preceding the start of citrate production, and it has been suggested that it is the accumulation of malate, which triggers citrate production through the action of the malate-citrate antiporter. The limiting factor for WT cytosolic malate accumulation could depend on cytosolic NADH, or if the original fungal enzyme had a low affinity or capacity to begin with. However, the regulation of *A. niger mdh* has not been studied in detail, and it might therefore be limited at the mRNA level. The direction of flux from the oxaloacetate node also depends on the relative Km-values of *OAH* and *Mdh*.

In support of the theory that citrate production is initiated by an increased cytosolic malate concentration (Rörh and Kubicek, 1981), we found that the *Mdh2* expressing mutant led to an increase in the initial citrate production rate compared with all the other

tested transformants (0.1 g/L.h vs. 0.08 g/L.h for the second highest strain Frds(V)-Fum1s). The *Mdh2* expressing strain, however, had a slower overall citrate production rate compared to the other mutants in the study. This suggests that, although malate is involved in the initiation of citrate production, a lack of cytosolic NADH, or low pyruvate carboxylase activity, probably limits the activity of malate dehydrogenase, and therefore the rate of citrate production through the citrate-malate antiporter.

7.5.2 Increased citrate productivity through the insertion of a cytosolic fumarase

The expression of a truncated form of *S. cerevisiae fum1*, which results in a targeted increase in cytosolic fumarase activity (Peleg *et al.*, 1990), leads to drastically increased citrate productivities in *A. niger*. From figure 5 it can also be seen that there is a very strong correlation between citrate production rate and fumarate consumption rate towards the later stages of the fermentation. The rate of fumarate consumption was too low to contribute to the production rate of citrate directly. It does however show the dependence of the fumarase and fumarate reductase genes on fumarate, indicating that it is indeed the substrate for both these enzymes. This correlation was not seen for the *mdh2* expressing strain, indicating that, as expected, the citrate enhancing effects of *mdh2* is not influenced by fumarate. It appears that, similarly to what has been reported for yeast (Pines *et al.*, 1997), the overexpresion of a cytosolicly targeted form of fumarase leads to the conversion of cytosolic fumarate to malate. The Km of yeast Fum1 is reported to be 13 fold lower for malate than for fumarate, and the result is therefore not unexpected.

Increased citrate productivity, similar to that observed for *fum1s* expression, was achieved through the expression of a truncated, cytosolic targeted form of the *Rhyzopus oryzae* fumarase (*fumR*) (Friedberg *et al.*, 1995). In *R. oryzae* the overexpresion of *fumR* results in increased fumarate productivity. The reason the same effect is not observed here, is most likely related to the very specific conditions required for fumarate production. Very high concentrations of calcium carbonate is required to neutralize the medium, and the fermentation proceeds at pH 5 (Friedberg *et al.*, 1995), compared to the low pH conditions applied in this study. The lack of malate as substrate for *fumR* therefore results in fumarate convertion to malate, even though its affinity is not very high for fumarate.

7.5.3 Increased oxalate production

Oxalate production is significantly increased in the fumarase expressing strains compared to the wild type and the other mutant strains. Insertion of a cytosolic fumarase in *A. niger* appears to lead to the conversion of cytosolic fumarate to malate, and malate then serves as substrate for oxalate production through the consecutive action of *Mdh* and *OAH*.

7.5.4 Succinate as substrate for mitochondrial citrate antiport

Expression of yeast *Frds1* results in an increase in the citrate productivity, which

is even higher than were seen for the fumarase expressing strains. It appears that fumarate is exported from the mitochondria to provide the substrate for the cytosolic fumarate reductase, and that the formed succinate then serves as substrate to the mitochondrial citrate antiporter. The structure and sequence similarities observed between the yeast CTP1p and DIC1p transporters suggests that the affinity of the cytosolic facing part of the citrate antiporter would have a similar affinity profile for different dicarboxylic organic acids as those, which have been reported for yeast DIC (Sandor *et al.*, 1994).This would imply that, although malate is the preferred substrate, succinate can be used for the mitochondrial antiport of citrate.

7.5.5 Co-overexpresion of fumarase and fumarate reductase in the cytosol.

To test if *fum1* or *fumR* and *frds1* expression in the same strain could have additive beneficial effects, the best citrate producing *frds1*overexpressing strain, frdsV, was transformed with *fum1s* and *fumRs*. From figure 3 it is clear that a combination of *fumRs* and *Frds1* leads to a further increased citrate production in the later stages of the fermentation, when compared to either of the single gene expression mutants. The co-expression of *fumR* and *Frds1* forms a cytosolic pathway from malate towards succinate, resulting in a higher concentration of succinate in the cytosol. The succinate can then be used by the citrate antiporter for enhancing citrate production. However, this is not true when *fum1s* is combined with *Frds1*. The very low affinity of *fum1s* for malate makes it unlikely that it would lead to the formation of a pathway towards succinate in the cytosol. The net effect would therefore be a competition for fumarate in the cytosol, with no additive advantage for citrate production. Another reason could be a lower expression level of the *Fum1s* gene compared to the *FumRs* gene in these two strains. This would make it more difficult to observe the effects of *Fum1s* in the *Frds1* expression background strain.

The citrate yield on glucose was also significantly increased in the double expression mutant, to an impressive 0.9 g citrate per g glucose in Frds(V)-FumRs1 during the later stages of the fermentation. The presence of trace amounts of Mn^{2+} in the medium (introduced through glucose, which had not been treated with an anion adsorption resin as is normally done for citrate production) makes this yield particularly impressive, as even 1 ppb Mn^{2+} can lead to up to a 10% reduction in yield (Mattey M., 1992).

The direct correlation between the glycolytic flux and citrate production rate was demonstrated by the strong exponential correlation ($R^2 = 0.92$) between citrate production rate and glucose uptake rate between the transformants. However, Frds(V)FumRs 1 does not correspond to this trend, as it has a higher citrate production rate than the trend suggest should be observed for its glucose consumption rate. This suggests that the dual insertion of *Frds1* and *fumRs* fundamentally improves the yield on citrate, which can clearly be observed in figure 4 (90% yield). It has been proven that a citrate yield in excess of 67% cannot be attained without fixing the CO_2 produced in the TCA cycle (Ruijter G.J.G., Kubicek C.P., and Visser J., 2002). The CO_2 is fixed through the action of pyruvate carboxylase (Ruijter G.J.G., Kubicek C.P., and Visser J., 2002), and it can be argued that the pathway, which has been created towards succinate, enables higher flux

through pyruvate carboxylase. The increased activity could be resulting from two effects: 1) an increased demand for the pyruvate carboxylase product, OAA, as it is a precursor of the inserted pathway; and 2) a positive effect on the redox balancing established through the action of *frds1*. *Frds1* has been demonstrated to be essential for re-oxidizing NADH to NAD^+ in *S. cerevisiae* (Enomoto *et al.*, 2002) under anaerobic condition. This would increase the flux though pyruvate carboxylase by helping to maintain the redox balance, which is strained by the overproduction of NADH under citrate overproduction conditions.

In this paper we provide supporting evidence for the role of cytosolic malate, and the importance of cytosolic malate dehydrogenase, in the initiation of citrate production. We also demonstrate several novel methods to enhance citrate production through insertion of heterologous genes. Furthermore, the gene insertions allowed citrate production under condition where the wild type and the control could not produce citrate.

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Chapter 8. Development of a novel reversible marker system for advanced gene-targeting in *A. niger*

8.1 Introduction

A. niger is intensively utilized by the biotechnology industry for enzyme and organic acid producton. The largest market for any of these products is for citric acid, with a global market of more than 900 000 tons per year (see chapter 2).

Several successful strain improvements (decreased by-product formation, enhanced resistance to factors negatively influencing production, and improved productivity) have been achieved to date, and demonstrate the potential of advanced targeted metabolic engineering strategies in *A. niger*. The recent release of a 4X coverage of the *A. niger* genome, and continued efforts by the American Department of Energy to provide a 9X coverage, is opening up a whole field of opportunities. Unfortunately, there are still only a limited number of published methods available for advanced genomic manipulations in *A. niger* (see chapter 3, table 3.2). It is therefore critical that the current state of gene-targeting technology be improved if full advantage is to be taken of these advancements.

The use of bipartite gene-targeting substrates (which will be referred to as the bipartite gene-targeting technique) and recyclable auxotrophic markers (henceforth referred to as the recyclable marker technique) for precise genome manipulation were initially developed in *S. cerevisiae* (Erdeniz *et al.*, 1997), but has been shown to work well in the model filamentous organism, *A. nidulans* (Michael L.Nielsen *et al.*, 2005). Employing a recyclable auxotrophic marker allows multiple gene manipulations to be performed without the need for, and limitations of, relying on multiple auxotrophies or dominant markers. The recyclable auxotrophic marker technique also has the added advantage of allowing for the creation of gene-targeting fragments using only PCR techniques, alleviating the need for cumbersome bacterial cloning steps. This work is the first instance of applying the recyclable marker and bi-partite gene-targeting techniques in the industrially relevant filamentous fungus: *A. niger*.

Homologous recombination events are rare in filamentous fungi, with non-homologous enjoining being the main pathway for double strand break repair, which is in contrast to yeast where homologous recombination is the main pathway for double strand break repair. This predominance of end-joining over homologous recombination leads to very low gene-targeting efficiencies in filamentous fungi. Fortunately, the bi-partite gene-targeting technique has been shown to enhance the efficiency of gene-targeting events in *A. nidulans* (Michael L.Nielsen *et al.*, 2005).

Industrial *A. niger* strains are very good candidates for advanced gene-targeting techniques. Many years of traditional strain improvement programs have resulted in strains with exceptional abilities of citrate overproduction, which must not be adversely affected by subsequent genetic manipulation activities. This prerequisite calls for techniques, which manipulates the strain in the intended manner (for instance: decrease by-product formation), but does not result in any pleiotropic effects.

Traditional gene deletion techniques result in the replacement of the gene of interest with an auxotrophic marker gene. This can be problematic as it is suspected that different expression levels are observed depending on the locus of integration in the genome. This variability, resulting from integrating the auxotrophic marker in a locus removed from the native marker locus, could lead to undesirable pleiotropic effects in the production host. This problem can be overcome by excising the marker used during the genomic manipulation, and restoring native marker gene. This is usually accomplished through an additional transformation step. The creation of several strains during a metabolic engineering improvement program, containing multiple genomic manipulations, therefore each has to have the native marker restored. During a strain improvement program this results in n x 2 transformations having to be performed (where n is the number of genomic manipulations attempted).

A new concept is therefore introduced here, which comprises the creation and use of a reversible auxotrophic marker strain. In principle an unlimited number of consecutive targeted genetic manipulations can be created using a reversible auxotrophic marker strain combined with the recyclable marker approach. This approach would then result in n + 1 transformations, compared to the n x 2 of a traditional strain improvement program.

8.2 Materials and methods

The *A. niger* wild type strain, N402, was used for all gene deletion and disruption experiments. The strain is a mutant with short conidiophores, compared to the wild type (N400).

8.2.1. Growth and selective media

Solid minimal media (MM) was made according to (Barratt *et al.*, 1965). Hygromycin was supplied by Sigma and minimal media containing hygromycin (MM-HYG) was prepared by adding 300µg/ml hygromycin to MM media after it had cooled to 50°C after autoclavation. Minimal medium containing uracil and uridine (MM-U&U) had 10mM uracil and 10mM uridine added by sterile filtration when the media had cooled to 50°C. Minimal media containing 10g/L acetate as sole carbon source is referred to as MM-Ace. MM-Ace-U&U is the same as MM-U&U except that glucose is replace with acetate, while MM-HYG-U&U is the same as MM-U&U with 300µg/ml hygromycin added at 50°C after autoclaving. Propionate plates are similar to MM plates, except that only 0.1% glucose (1g/L) instead of 10g/L were used, and between 16 and 40g/L propionate (concentration indicated in text) was added. Priopionate-U&U plates have 10mM uracil and 10 uridine added as well. 5-fluoroorotic acid (5-FOA) was obtained from Sigma. As this compound is temperature sensitive 5-FOA medium was therefore made by adding dissolved 5-FOA through a sterile filter to the desired concentration of 1.3 mg/ml to minimal medium MM-U&U cooled to 50°C after autoclaving.

8.2.2 Plasmids, Primers and PCR

Two plasmids were used in this study as substrates for the generation of the marker regions of the bi-partite fragments. Plasmid pDel1 contains *pyr-4* of *N. crassa* flanked by 300b direct repeats (Michael L.Nielsen *et al.*, 2005) and plasmid pAN7-1 harbors the bacterial *hph* gene, conferring hygromycin resistance, between a strong fungal promoter (*gpdA*) and the *trpC* terminator [(Tolsters A., 2003);(Punt *et al.*, 1987)].

Oligonucleotides were supplied by MWG-Biotech AG and are listed in Table 8.1. PCR was performed using the Expand High Fidelity PCR kit from Roche according to manufacturer's recommendations. All PCR products were purified from agarose gels using a GFX™ purification kit (Amersham Pharmacia).

Table 8. 1. Primers used in this study in the construction of the bi-partite targeting fragments

Primer	Sequence
P1	GGAACTGCCTTTGCAGGTGTGGC
P2	GATCCCCGGGAATTGCCATG-CACAAAGTCCTCCTCATCCGAAG[1]
P2.2	GATCCCCGGGAATTGCCATG-CAAGATCAACAGACCACGTTCGGG
P3	GGACTGAGTAGCCTGACATC-CACTAAGGAGCTACTAGATCTTGC[2]
P4	CACCCTATAATAGCCTGCAGGATC
H1	CATGGCAATTCCCGGGGATC-GCTGATTCTGGAGTGACCCAGAG[1]
H2	CTGCTGCTCCATACAAGCCAACC
H3	GACATTGGGGAATTCAGCGAGAG
H4	GATGTCAGGCTACTCAGTCC-CGTTGTAAAACGACGGCCAGTGC[2]
F1	GCCTTTGGTGGAAGTTGTTCC
F2	GATCCCCGGGAATTGCCATG-GACCAGTTAAGACGGGTCTGG[3]
F3	ATTCCAGCTGACCACCATG-GGGACTACCAGTTTTCTCATG[4]
F4	CTAGTCTAGCTTTCGGAGCAC
Y1	CATGGCAATTCCCGGGGATC-TGGATAACCGTATTACCGCC[3]
Y2	GTTGTCTGCTTGCGCTTCTTC
Y3	CCAGAAGCAGTACACGGC
Y4	CATGGTGGTCAGCTGGAAT-TGCCAAGCTTAACGCGTACC[4]
Z1	CATTCGTTCATTGATCCACCGC
Z2	CGGCTTGTGGCGCTGATAAC

[1] The initial part of the sequence, separated by hyphen from the second section, is the sequence of the adaptomere (adaptomere A in figure 8.1) added to enable a fusion PCR reaction to be employed to create the final gene-targeting fragment.

[2] The initial section of sequence represents adaptomere B in figure 8.1

[3] The initial section of sequence represents adaptomere A in figure 8.2

[4] The initial section of sequence represents adaptomere B in figure 8.2

8.2.3 Gene-targeting substrates for reversible disruption of native A .niger pyrG

PCR was used to amplify the two marker-containing fragments of the bipartite substrates for the loss of function disruption of *pyrG* using pAN7-1 as template. The primer pairs used were: (H1 and H2) and (H3 and H4) (see figure 8.1).

The reversible loss of function disruption of *pyrG* coding sequence (1500bp) in the genome, was achieved by using the upstream 2/3 (1000bp) of the *pyrG* coding sequence, and downstream 2/3 (1000bp) of the *pyrG* coding sequence, as specific targeting fragments. Genomic *pyrG* was used as template for the PCR reaction, using primer sets: (P1 and P2) and (P3 and P4). The upstream targeting fragment was fused to the upstream *hph* marker fragment, while the downstream targeting fragment was fused to the downstream *hph* marker fragment, using primer sets: (P1 and H2) and (H3 and P4), respectively. After correct integration in the genome this creates a direct repeat of 500bp, of the middle third of *pyrG,* on either side of the *hph* marker cassette (see figure 8.1).
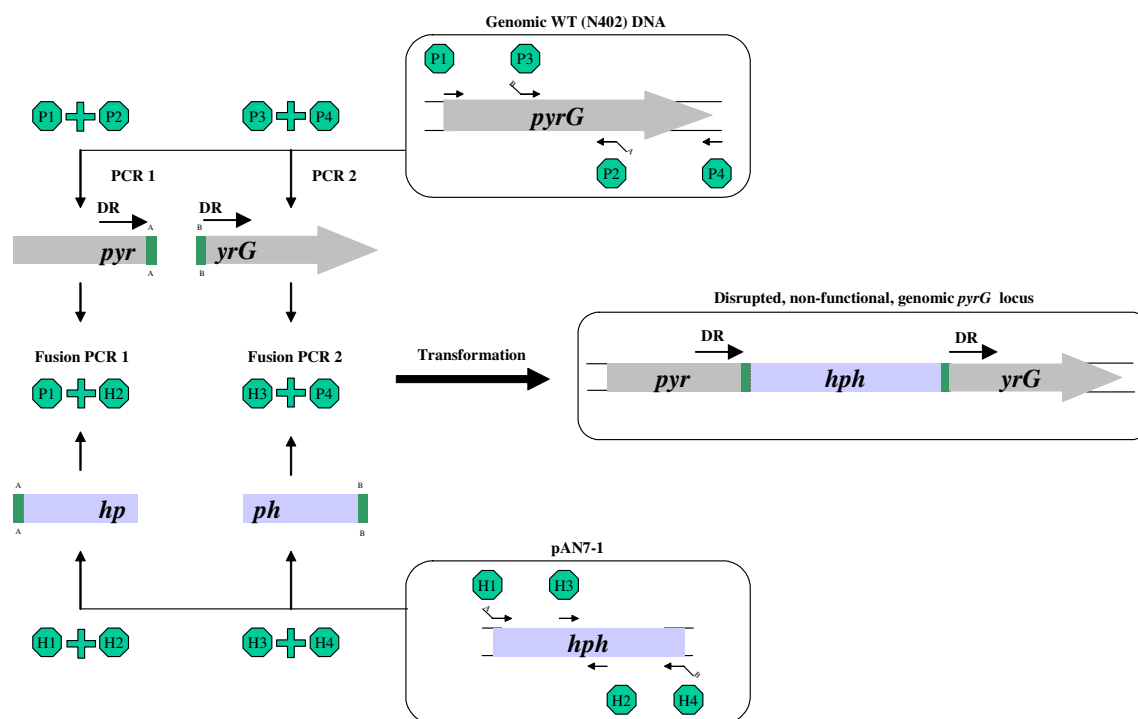
Figure 8.1. The PCRs and templates involved in the creation of the bi-partite targeting substrates for the loss of function disruption of *pyrG*. The non-functional genomic *pyrG*, as it would appear after HR of the bi-partite fragments with each other and integration into the native *pyrG* locus, is also depicted. The arrows marked DR above the *pyrG* targeting substates represents the areas of perfect homology between the two fragments, which will later be employed to pop-out the *hph* gene. The regions marked with a small A and B refers to the position of the primer adaptomeres (see table 8.1) in the constructs.

8.2.4. Gene-targeting substrates for deletion of acuB using the pyr-4 recyclable marker

PCR was used to amplify the two marker-containing fragments of the bipartite substrates for recyclable deletions, using pDEL1 as template (see Figure 8.2), similarly to Nielsen *et al.* (Michael L.Nielsen *et al.*, 2005). The primer pairs (Y1 and Y2) and (Y3 and Y4), respectively, were used for the *pyr-4* upstream and downstream marker fragments in the *acuB* deletion (see figure 8.2).

The deletion of *acuB* in the genome uses the PCR amplified, upstream (1.1 kb) and downstream (1.3 kb) specific targeting sequences flanking *acuB*. Genomic DNA was used as template, and the primer pairs (F1 and F2) and (F3 and F4), were employed respectively. The specific upstream targeting sequence was fused to the *pyr-4* upstream marker fragment by PCR using primers F1 and Y2, while the specific downstream targeting sequence was fused to the *pyr-4* downstream marker fragment by using primers Y3 and F4 (see figure 8.2).
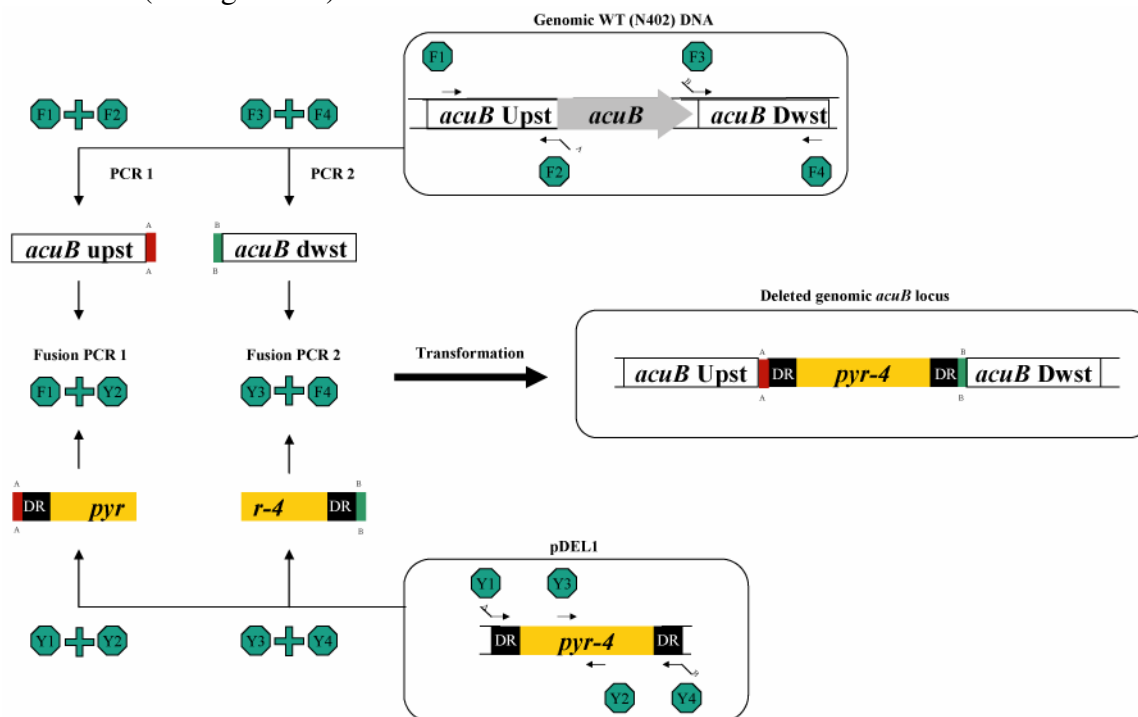
Figure 8.2. The PCRs and templates involved in the creation of the bi-partite targeting substrates for the deletion of *acuB* using the *pyr-4* recyclable auxotrophic marker. The deleted genomic *acuB locus*, with *acuB* replaced by the recyclable *pyr-4* deletion cassette, is also depicted. The regions marked DR in the targeting substates represents the areas of perfect homology between the two fragments, which will later be employed to pop-out the *pyr-4* gene. The regions marked with a small A and B refers to the position of the primer adaptomeres (see table 8.1) in the constructs.

8.2.5. Fungal Transformations

Genetic transformation of *A. niger* protoplasts was preformed similarly to the transformation of *A nidulans* by Nielsen *et al (Michael L.Nielsen et al., 2005)*. All transformations were performed with 1-2*10^{6} protoplasts in 100 μl transformation buffer (Johnstone, 1985). For these experiments 1-2 μg of each DNA fragment were used unless otherwise indicated.

8.2.5.1 Selection of pyrG disruptants

Transfomants for the *pyrG* disruption were selected by plating the transformed protoplasts in selective minimal medium containing 1M sucrose, hygromycin at 500μg/ml, 10mM Uracil and 10mM Uridine . The resulting colonies were purified on MM-HYG-U&U media by streaking out spores to obtain single colonies. The purified colonies were then tested for the ability to grow on 5-FOA plates and inability to grow on MM plates.

8.2.5.2 Selection of ΔacuB transformants and pop-out of recyclable pyr-4 marker

The transformed protoplasts were plated in 1M sucrose minimal medium containing 300μg/ml hygromycin. The resulting colonies were then replica-plated onto 4% propionate, 0.1% glucose minimal medium. Colonies with the ability to sporulate on this medium were then streak-purified on MM-HYG.

10^6 spores from some colonies presenting the correct phenotype, which was defined as a lack of growth on MM-Ace and growth on MM-HYG, were then plated on 5-FOA plates to select for pop-out of the recyclable *pyr-4* marker.

8.2.5.3 Reconstitution of pyrG

Endogenous *pyrG* was reconstituted by plating on minimal media 10^6 spores of streak-purified *ΔacuB* transformants after 5-FOA pop-out. The resulting colonies were then tested to ensure that the hygromycin resistance was lost (the result of *hph* pop-out), and the inability to grow on MM-Ace was preserved (resulting from the deletion of *acuB*).

8.2.6. Southern blot hybridization

Approximately 2μg of genomic DNA were isolated similarly to Nielsen *et al.* (Nielsen *et al.* 2005) and loaded on gels. The DNA from the different strains were digested with restriction enzymes as described in the relevant sections. The *pyrG* locus was detected in the first (figure 8.4 and 8.5) and second (figure 8.6 and 8.7) Southern blots by probing with the 500bp direct repeat of *pyrG* coding sequence surrounding the hygromycin resistance marker in the disruption cassette of *pyrG* (see figure 8.1). The probe was created by PCR from genomic DNA using primer pair: P3 and P2. The first blot was simultaneously probed for the hygromycin resistance cassette with the 500bp of the *hph* gene present in both bi-partite gene-targeting fragments (see figure 8.1), primer pair H3 and H2 was used to PCR the probe from plasmid pAN7-1. The second (figure8.4 and 8.5) and third (figure 8.9) Southern blots were created in duplicate from two identically run and loaded gels. The third blot was probed for the *acuB* gene with a 1kb fragment of the coding sequence of the *acuB* gene obtained by PCR from genomic DNA using primer pair: Z1 and Z2.

Blotting was done according to the method described by Sambrook and Russell (Sambrook and Russell, 2001) and using RapidHybe™ hybridization buffer (Amersham Pharmacia) for probing. All probes were radioactively labeled with α-^{32}P-dCTP by random priming using Rediprime II™ kit (Amersham Pharmacia).

8.3. Results

The purpose of this work was to create a process through which a strain of interest can be transformed to auxotrophy in a reversible manner, such that it can be used as a background strain for genomic manipulations, and afterwards reverted to wild-type prototrophy. The only prerequisites for the parental strain are that it must be pyrimidine prototrophic, and sensitive to hygromycin. Once the R-AUX strain has been created it can be used as a normal pyrimidine auxotrophic strain. However, it is also important that the auxotrophic marker used for further transformations in the reversible auxotrophic background be recyclable, as this will enable multiple deletions to be attempted. Such a reversible auxotrophic (R-AUX) strain was created, and a recyclable marker developed for *A. nidulans* was used to do a proof-of-principle deletion in this strain background. The deletion strain was then restored to wild-type prototrophy, and the whole process tested by southern bloting. The process is outlined in figure 8.3 as a flow diagram illustrating the steps involved in creating and using the R-AUX background.

8.3.1 Reversible, loss of function, disruption of pyrG in A. niger WT strain N402

The disruption of *pyrG* was achieved using the bipartite gene-targeting technique. Firstly, the four component PCR fragments were created. These PCR fragments were then assembled through fusion PCR to form the bipartite gene-targeting substrates. The two fragments to be used for targeting the genomic locus consisted of: a 1kbp upstream fragment representing the upstream 2/3 of *pyrG* coding sequence, and a 1kbp downstream fragment representing the downstream 2/3 of *pyrG* coding sequence. Next, two partial marker fragments were created by PCR, using the pAN7-1 plasmid as template. The upstream marker fragment consists of a 1.2kbp fragment, representing the inactive, upstream 2/3 of the *hph* marker cassette. A 1.3kbp downstream marker fragment, representing the inactive, downstream 2/3 of the *hph* marker cassette, was also created. To create the two transformation fragments the upstream and downstream *pyrG* targeting fragments were joined to the up- and downstream hygromycin resistance marker fragments, respectively, by fusion PCR using two different sets of adaptomeres incorporated during the original PCR. The two inactive sections of the marker combine through HR during transformation to reconstitute an active hygromycin resistance marker cassette, which was used for selection of the transformants.

Transformation results in the *in vivo* replacement of the final 1/3 (500bp) of the *pyrG* coding sequence with a 3.0kbp hygromycin resistance marker cassette, followed by the final 2/3 (1000bp) of *pyrG* (see figure 8.1). This leads to the disruption of *pyrG* activity, and the creation of a 500bp direct repeat around the hygromycin resistance marker cassette. This direct repeat later allows the excision of the hygromycin marker cassette, and thus reconstitution of the native *pyrG* gene.

The potential *pyrG* disrupted transformants were scored on 5-FOA plates, containing 300 µg/ml hygromycin, and it was found that 30 out of 50 tested colonies were able to grow, while being unable to grow on MM plates.

Figure 8.3. The flowchart demonstrates the steps involved in the creation and use of a reversible pyrimidine auxotrophic strain as background for genomic manipulations (e.g. deletions, GFP-tagging, promoter replacement, ext.). The first step in the process is to create the reversible auxotrophic marker in the particular strain of interest (STAGE A) for a specific strain improvement program (see section 3.1). A recyclable auxotrophic marker (*N. crassa pyr-4*) can then be used to do genomic manipulations (see section 3.5). Once the initial genomic manipulations have been achieved, the recyclable auxotrophic marker is poped-out (see section 3.6). Poping-out the *pyr-4* marker restores the strain to auxotrophy, which allows the user to either do another round of genomic manipulations using the same *pyr-4* recyclable marker (STAGE B), or restoring the strain to native prototrophy (see section 3.7), (STAGE C). The new manipulated strain is now ready for further study of the particular manipulations, which have been introduced.

Parental strain
pyrimidine (pyrg+)
prototrophic

STAGE A

Creation of reversable pyrimidine auxotrophic strain
through the loss of function disruption of *pyrG*, using *hph* (hygromycin resistance marker) for selection.

Use recyclable *pyr-4* auxotrophic marker to manipulate the genome,
transforming the strain to pyrimidine prototrophy

Pop-out *pyr-4*
by plating the spores on 5-FOA, and restore strain to auxotrophy.

YES

Is another genomic manipulation required?

STAGE B

NO

STAGE C

Restore wild-type pyrimidine prototrophy in mutant strain
by poping-out the *hph* marker through plating the spores on MM.

8.3.2 The pyrG auxotrophy is reversible

The reversibility of the *pyrG* disruption was illustrated by pop-out of the *hph* marker through plating spores on MM, lacking uridine and uracil. The resulting colonies were then streak purified and scored for growth on MM, and lack of growth on MM-HYG. The tested colonies were found to reconstitute *pyrG* to native activity, with concomitant loss of hygromycin resistance.

Southern blot analysis were used to ensure that the native and disrupted *pyrG* loci had the expected configuration in the colonies showing the expected phenotype. The results of the Southern blot have been separated into two figures to ease interpretation. Figure 8.4 contains the analysis of the disruption of *pyrG* in the R-AUX strain, while figure 8.5 depicts the study of the strain in which pyrG was functionally reconstituted. As can be seen from figure 8.4 and figure 8.5, no ectopic integrations occurred and the *pyrG* gene can be functionally reconstituted in the reversible auxotrophic strain.
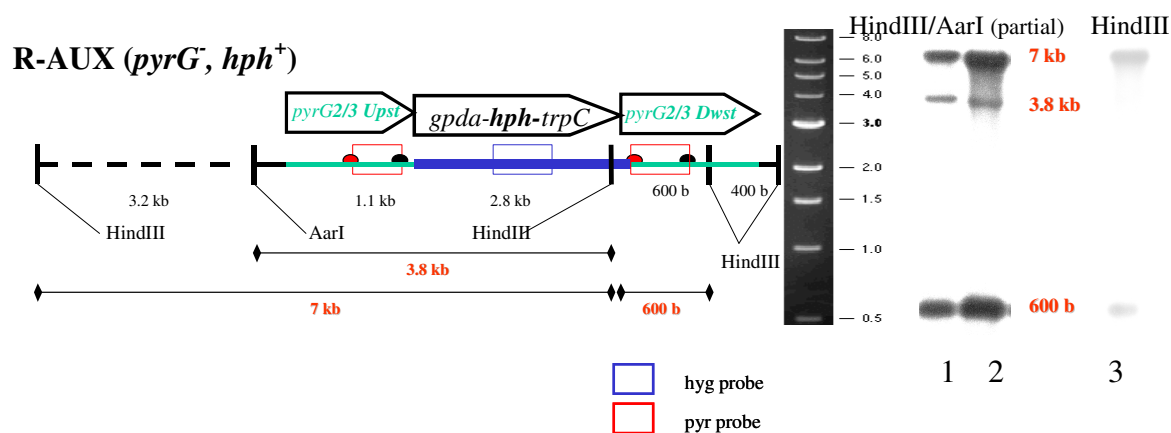
Figure 8.4. The *pyrG* locus disrupted by the *hph* marker cassette, including restriction enzyme recognition sites, of the R-AUX transformant is depicted. The observed digestion pattern detected by southern blot is also included in the figure (a 1kb NewEngland Biosciences DNA ladder is included for reference). Two probes (targeting the *hph* cassette, or the 500bp repeat in *pyrG* surrounding the *hph* cassette) were used simultaneously to be able to detect all possible integrations of the DNA substrates used for transformation. Two different digestions were performed: The first digestion, lanes 1 and 2, was performed on duplicate samples of R-AUX, using *HindIII* and *AarI* restriction enzymes, which resulted in three bands (7kbp, 3.8 kbp and 600bp). The 7kbp band corresponds to a fragment undigested by *AarI*, which results because of the low efficiency of *AarI*. The 3.8kbp band can be seen from the figure to exactly correspond to the expected *HindIII/AarI* digestion patern, while the 600bp band is expected regardless of *AarI* activity when *HindIII* is used. The second digestion, lane 3, was performed using only *HindIII* and resulted in two bands at 7kbp and 600bp. All the bands detected in the southern blot correspond to the expected digestion pattern, which shows that the R-AUX strain contains only the expected disruption of *pyrG* without any ectopic or unwanted integration events.
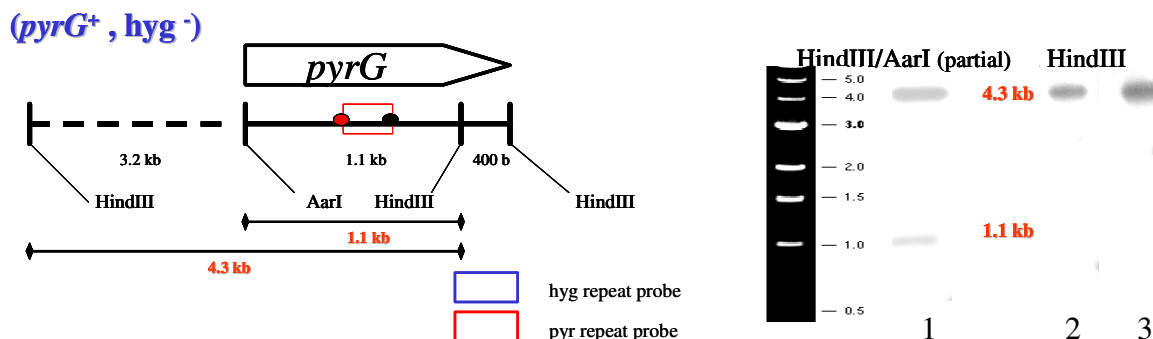
Figure 8.5. The reconstituted *pyrG* locus in the R-AUX background, as well as the WT *pyrG* locus is depicted in this figure. As was done in figure 8.4, two probes where used simultaneously to be able to detect all possible integrations of the DNA substrates used for transformation. The three samples analysed in this figure is R-AUX in lane 1 and 2, and a WT sample in lane 3. The expected digestion pattern for this locus is identical to that of the wild-type, since the reversible auxotrophy was designed to revert to the wild-type sequence upon *hph* marker cassette pop-out. The bands detected by southern blot were: 4.3kbp and 1.1kbp for the *HindIII/AarI* partial digestion of R-AUX and 4..3kbp for the *HindIII* digestion of R-AUX and the WT. As was the case in figure 8.4, the extra 4.3kbp band detected for the *HindIII/AarI* digestion results from the partial digestion of the genomic DNA by AarI, while the 1.1 kbp band results when both *HindIII* and *AarI* digestion occurred. Only the expected bands were detected, demonstrating the correct pop-out of the *hph* marker cassette and reconstitution of native *pyrG*.

8.3.3 Testing the stability of the PyrG disruption in the reversible auxotrophic strain

The stability of the reversible auxotrophy was determined by plating different concentrations of spores on MM. It was found that 1 spore in 10 000 will have spontaneously reverted to native *pyrG* function (if the tested spores were harvested from a plate grown for 4-5 days after inoculation from a -80°C stock). A similar number of false positive transformants (presenting the expected phenotype of *pyrG* prototrophy, but lacking actual integration of a marker), around 100 colonies per 1 million protoplasts used for transformation, were found when protoplasts were tested. The reconstitution of *pyrG* represents a stochastic (non-normal distribution) event and a very large number of experiments would therefore be required to obtain an accurate result. However, these tests served as a general indication of the challenges to be encountered when using the reversible auxotrophic strain in further deletion studies. It was also found that colonies with the ability to grow on MM-HYG plates, even though this should not be possible, were found if R-*AUX* spores were plated out on MM-HYG plates instead of MM plates. This will be discussed in more detail in section 4. These experiments indicated the need to enhance the stability of the reversible auxotrophy, since a background of 100 colonies per 1E6 protoplasts would represent a large amount of screening to find the correct transformants.

8.3.4 Enhancing strain stability of the reversible auxotrophic strain

The reversible auxotrophic strain has been shown to produce false positives during transformation. The false positives have the ability to grow on MM, lacking uracil and uridine, but containing hygromycin. It was thought that enhancing the stability of the hygromycin resistance cassette and *pyrG* disruption would diminish this problem. The length of the direct repeat surrounding the *hph* marker cassette was therefore shortened in an effort to decrease the background during transformation. This was achieved by creating new upstream transformation fragments (primer pair P1 and P2.2: see table 8. 1). The same approach was employed as was explained above for the original *pyrG* disruption, but instead of using a 1kbp upstream targeting fragment, a 900bp fragment lacking the final 100bp compared to the original upstream targeting fragment was used. This newly created strain displayed a similar digestion pattern in a Southern blot assay compared to the original reversible auxotrophic strain. This indicated the lack of ectopic integrations or other unwanted integration patterns in the newly created strain, and showed that it could be used to compare the effect on strain stability of shortening the direct repeat length. (see figure 8.6).
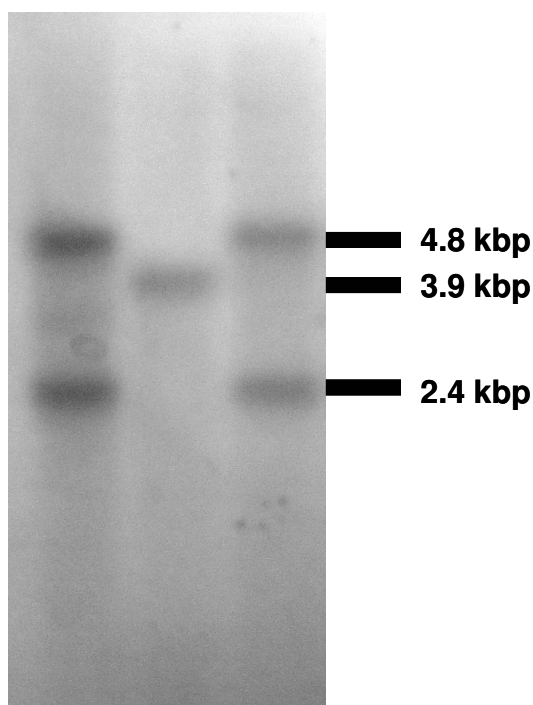
Figure 8.6. Southern blot probed with a 500bp fragment of *pyrG*, showing the similar digestion patterns of the R-AUX strain with a 500bp repeat and the new strain containing the shortened 400bp repeat (lane 1 and 3, respectively). Lane 2 shows the band created for the WT, non-disrupted, *pyrG*. The new R-AUX strain therefore had no ectopic integrations, and only contains the correctly integrated *hph* marker cassette.

A reduction in direct repeat length of 100bp, from 500bp to 400bp, was found to enhance strain stability, and led to a decrease in the number of false positive transformants by approximately half (assayed by separately plating spores and protoplasts onto MM lacking hygromycin and recording the number of viable colonies per 1E6 spores or protoplasts plated, results not shown).

8.3.5 Use of recyclable marker for the deletion of acuB in the reversible auxotrophic parent strain

As it was now proven that the R-AUX strain could be reconstituted to native *pyrG* function, and could therefore serve as a host strain for further deletions, a further deletion of *acuB* was attempted in this background as a proof-of-concept example. *AcuB* is a global regulatory gene responsible for inducing several gluconeogenic genes (see chapter 9), and the disruption of *acuB* has been found to lead to an inability to grow on acetate as sole carbon source [(Sealy-Lewis and Fairhurst, 1998),(Ruijter G.J. *et al.*, 1999)]. The bi-partite method was once again employed to create the deletion fragments, but, now a cassette containing a recyclable pyrimidine auxotrophy marker, *Neospora crassa pyr-4,* flanked by 300b direct repeats (Nielsen M. L. *et al.* 2005), was inserted in the R-AUX background, and in such a way as to delete the whole of *acuB* (see figure 8.2). The bi-partite gene-targeting substrates were created through several steps. Firstly, the gene-targeting fragments were creates using a 1.1kbp section of chromosomal DNA from the upstream region preceding *acuB* as template for the upstream targeting fragment, as well as using the 1.2kbp region of chromosomal DNA downstream of *acuB* as template for the downstream targeting fragment. Next, the two fragments of the auxotrophic curing marker were created by PCR using plasmid pDel1 as substrate, and each contained an inactive 2/3 of the marker cassette (see figure 8.2). The up- and downstream fragments were then fused to the up- and downstream marker fragments through fusion PCR. The two gene-targeting substrates combined *in vivo* to a functional marker through HR, replacing *acuB,* and allowing selection for pyrimidine prototrophic transformants.

The transformants (~250) were screened for the *acuB* deletion by replica plating the transformation plates onto 4% propionate, 0.1% glucose plates. Propionate interferes with sporulation and growth of strains with the ability to use acetate (Sealy-Lewis and Fairhurst, 1998), and it was therefore possible to discern the Δ*acuB* colonies by their ability to sporulate and their enhanced growth. The sporulating colonies were then scored by growth on MM, MM-HYG, MM-Ace and 1.6% Propionate plates. The colonies showing severely retarded growth on MM-Ace compared to the control N402 strain, as well as the ability to grow on MM-HYG, MM and propionate plates, were considered possible positive transformants. Ten potential colonies were scored in this way, of which 6 displayed the correct phenotype.

8.3.6 Pop-out of recyclable marker

Spores taken from the 6 transformants showing the correct phenotype for a *acuB* deletion after being streak purified and grown on MM-HYG, were then plated on 5-FOA to select for the pop-out recombination of *pyr-4*. Two of the resulting colonies for each strain were then streak purified on MM-HYG-U&U plates, and scored for their ability to grow on MM-U&U plates, MM-HYG-U&U plates and their lack of or very poor growth on MM and MM-Ace-U&U, respectively. The expected phenotype was observed for all 12 of the purified pop-out strains tested in this way.

At this stage in the strain creation process it is possible to once again employ the recyclable *pyr-4* marker to manipulate the genome again, or reconstitute of the native *pyrG*. The latter was option was followed as the aim of this work was to do a proof-of-concept demonstration of the entire process.

8.3.7 Reconstitution of native pyrG

The last step in the process was to reconstitute the native *pyrG* gene in the Δ*acuB* strain. This was done by plating spores from the streak purified *pyr-4* excised strains on MM to pop-out the *hph* marker. The *hph* marker cannot be counter selected for by itself, but by selecting for the reconstitution of *pyrG*, *hph* is simultaneously popped-out. Twenty of the resulting colonies were scored for their ability to grow on MM, not grow on MM-HYG, and grow very poorly on MM-Ace, all of which displayed the expected phenotype.

8.3.8 Verification of the complete deletion and reconstitution cycle by Southern blotting

One purified representative strain from each phase of the process was tested by southern blotting, namely: WT (N402), Primary reversible auxothrophic disruption mutant (R-AUX), Δ*acuB* deletion mutant (R-AUX-Δ*acuB* (*pyr-4* $^+$)), Δ*acuB* deletion mutant with recyclable *pyr4* marker excised (R-AUX-Δ*acuB* (*pyr-4* $^-$)), and the final Δ*acuB* deletion mutant with native *pyrG* function restored (N402-Δ*acuB*). Two identical southern blots were made of these strains digested over night with XbaI. The first blot was probed with the 500bp repeat of *pyrG* (figure 8.7), while the second blot was probed with a 1kb PCR fragment of the *acuB* gene (figure 8.9). The blot probed with the 500bp repeat of *pyrG* showed that the *hph* disruption of *pyrG* gene remained stabile during the subsequent *pyr-4* deletion of *acuB* (R-AUX-Δ*acuB* (*pyr-4* $^+$)) and pop-out of *pyr-4* (R-AUX-Δ*acuB* (*pyr-4* $^-$)), and could be successfully reconstituted to its native *pyrG* sequence (N402-Δ*acuB*) (see figure 8.7). The only band in the southern blot probed with *pyrG*, which could not be identified (see figure 8.8 for a description of the expected digestion patterns of XbaI digested chromosomal DNA for strains containing either the native or reconstituted *pyrG* (A), or *pyrG* disrupted by the *hph* marker cassette(B)), was the 3.2kbp band detected in both R-AUX-Δ*acuB* (*pyr-4* $^+$) and R-AUX-Δ*acuB* (*pyr-4* $^-$). It is possible that this band is the result of the *pyrG* annealing to *pyr-4* in the deletion mutants, which would explain why it is not present in the final *pyrG* reconstituted strain (N402-Δ*acuB*). Although, this would still not explain why the band is present in R-AUX-Δ*acuB* (*pyr-4* $^-$)), which should not have *pyr-4* in the genome. However, this extra band is not of great importance, as it did not influence the expected growth phenotypes of the strains in any of the stages of the proof-of-concept deletion of *acuB*, and the final strain only contained the expected native *pyrG* band. This shows that even if this was a complex event influencing *pyrG* in the genome, no trace of it remains once the *hph* marker cassette was poped-out and *pyrG* was reconstituted.

The second blot (figure 8.9), which was probed with the PCR fragment of *acuB*, demonstrated that the *acuB* gene was successfully deleted. The final reconstituted deletion mutant therefore had native *pyrG* function, with *acuB* deleted.

Figure 8.7. Southern blot representing the creation of the R-AUX strain (lane 2), the proof-of-concept deletion of *acuB* in the R-AUX background using the recyclable *pyr-4* marker (lane 3), the strain with *pyr-4* popped-out (lane 4) and the eventual reconstitution of the native *pyrG* (5). The 500bp repeat of *pyrG*, surrounding the *hph* marker cassette, was used as probe to enable the visualisation of the effects of the various manipulations on the *pyrG* locus.
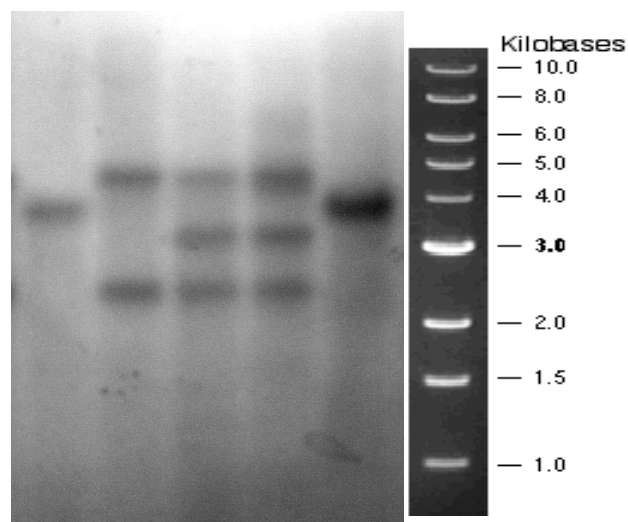
The lanes, from left to right:	[Detected band sizes]
1. WT (N402)	3.9 kbp
2. R-AUX	4.8 kbp and 2.4kbp
3. R-AUX-Δ*acuB* (*pyr-4* ⁺)	4.8 kbp, **3.2 kbp** and 2.4 kbp
4. R-AUX-Δ*acuB* (*pyr4* ⁻ pop-out)	4.8 kbp, **3.2 kbp** and 2.4 kbp
5. N402-Δ*acuB*	3.9 kbp
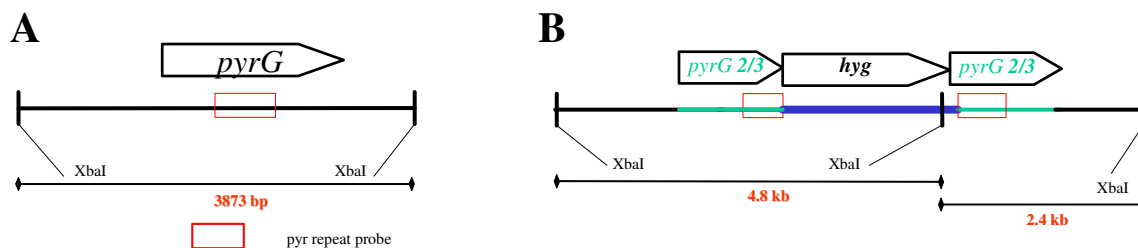
Figure 8.8. The figure shows the *pyrG* genomic locus as it would appear in (A) the WT or a reconstituted *pyrG* strain (N402-Δ*acuB*), (B) the R-AUX strain and the strains created in the R-AUX background with *pyrG* disrupted. The expected band sizes for a *XbaI* digestion are given for both cases: one 3.9 kbp band for the native *pyrG* strains, and two bands of 4.8kbp and 2.4kbp for the strains where *pyrG* is disrupted by the *hph* marker cassette. Note, in figure 8.8(B) the 4.8 kbp fragment will be shortened by 100bp to 4.7 kbp in the shortened repeat version of R-AUX (see section 3.4).
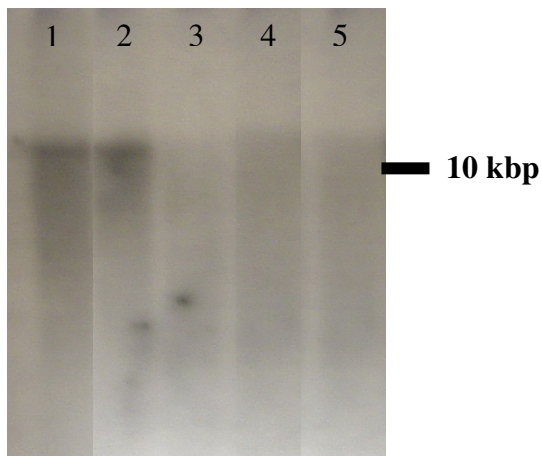
Figure 8.9. Southern blot using *XbaI* digested genomic DNA from: WT (lane 1), R-AUX (lane 2), N402-Δ*acuB* (lane 3), R-AUX-Δ*acuB-pyr4*⁻ (lane 4), R-AUX-Δ*acuB-pyrG*⁺ (lane 5). A 1 kbp fragment of the *acuB* gene was used as probe, a band would therefore be seen if *acuB* is present, and no band would be detected when *acuB* has been deleted. It can be seen that the R-AUX strain and the WT are the only strains containing *acuB*. No bands were detected for the 3 strains in which *acuB* was expected to be deleted.

8.4. Discussion

The major advantage of the reversible auxotrophic marker system is the ability to restore the organism to native prototrophy after several rounds of genetic manipulation. This allows for enhanced accuracy in the study of a specific genetic modification, minimizing the risk of pleiotropic effects introduced by a heterologous, or randomly introduced marker obscuring the results. Another advantage of the reversible auxotrophic marker system is the reduction in effort required when reconstituting the native marker activity during an industrial strain improvement program. During a strain improvement program the need to reconstitute the native marker after each genomic manipulation step to test the effect of the alteration on production, results in n x 2 transformations (where n is the number of genomic manipulations attempted). The same program would require only n+1 transformations, where the single extra transformation consists of the creation of the reversible auxotrophic marker strain in the appropriate strain background.

Currently there are only a limited number of transformation markers available for *A. niger*, which makes it difficult to do multiple consecutive genomic manipulations (see chapter 3, table 3.2). The use of the recyclable marker system from *A. nidulans* presents an alternative solution to multiple marker usage for multiple gene modifications, and allows for an almost unlimited number of consecutive modifications to be achieved. The ease of use of the selectable/ counter selectable *pyr-4* marker also makes it simple to score transformants and pop-out strains, which facilitates strain creation. The high percentage of correct integrations of the *pyrG* disruption fragments into the genome (60%) also supports the use of the bipartite system over the more traditional continuous fragment integration systems. It has been demonstrated in *A. nidulans* and *S. cerevisiae* that it is possible to do advanced strain manipulations, such as: promoter replacements, GFP tagging and allele replacement. The results presented in this work demonstrate that there should be no fundamental reason why these techniques cannot be used in *A. niger* through the recyclable marker system.

. The unexpected ability of a small percentage of spores from the reversible auxotrophic strain to grow on media lacking pyrimidines, but containing hygromycin, raises several questions. It suggests that, either multiple nuclei are present in the spores and can therefore contain nuclea with *pyrG* disrupted by the *hph* marker casette, and nuclea where *pyrG* has been reconstituted with concominant loss of the *hph* marker; or the colonies managed to achieve the combination of markers in the first few multiplications of the nuclei during germination, resulting in a heterokaryon. The finding that at least two strains of *A. niger* contains only one nucleus ((Yuill, 1950); (Shcherbakova *et al.*, 1978)) argues against the possibility of multiple nuclei in the spores of the reversible auxotrophic strain.

The potential problems for R-AUX as host strain for multiple rounds of genomic manipulations caused by the instability of the *hph* marker cassette were drastically reduced by decreasing the direct repeat length surrounding it. This demonstrates that the background observed during transformation was caused by the instability of the *hph* marker cassette disrupting *pyrG*. Importantly, it also demonstrates that the background experienced during transformation is not an insurmountable problem.

In conclusion, the reversible auxotrophic strain background, when combined with the bipartite and recyclable marker systems, offers an excellent advanced gene-targeting system to enhance any small- or large-scale detailed analysis of single or multiple genomic manipulations in *Aspergillus niger.*

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Chapter 9: Physiological characterization of an *acuB* deletion mutant

9.1. <u>Introduction</u>

The deletion of the global acetate metabolism regulatory gene (*acuB*) in *A. niger* was attempted with the aim of studying the effect of deleting this regulatory protein on organic acid production under glucose repressing conditions. Even though *acuB* is reported to be repressed directly by the main glucose repression gene, *CreA*, it was still suspected to have a basal level of activity. This basal level of expression was suspected since the glyoxalate bypass genes, which are induced by *acuB*, were shown to have a low level of activity under glucose repressive conditions (Ruijter G.J. *et al.*, 1999). Also, the lack of acetate formation during oxalate production (oxalate is formed by the enzymatic cleavage of OAA to oxalate and acetate) in a high glucose concentration medium, was seen as a possible indication of the role of *acuB*. It was also found that another gene product ACS (acetyl CoA synthetase), which is induced by *acuB*, had detectable enzyme activity levels under glucose repressing conditions in *A. niger,* but not in *A. nidulans* (Sealy-Lewis and Fairhurst, 1998). *AcuB,* which is itself induced by acetate, have been reported to have wide ranging regulatory roles, as can be seen from table 9.1.

Table 9.1. The reported regulatory roles of acuB in *A. niger,* and its homologues in *S. cerevisiae* (*cat8*) and *A. nidulans (facB)*, as well as the general regulation of these genes are represented in the following table.

Genes induced by *acuB*	Direct repression by *creA*	Glucose induced protein degradation	Organism	Author
acetylCoA synthase			*A. niger*	(Ruijter G.J. *et al.*, 1999) (Sealy-Lewis and Fairhurst, 1998)
NADP-isocitrate dehydrogenase	**YES**		*A. niger*	(Ruijter G.J. *et al.*, 1999) (Sealy-Lewis and Fairhurst, 1998)
isocitrate lyase	**YES**	**Reported in S. cerevisiae** (Ordiz *et al.*, 1995)	*A. niger*	(Ruijter G.J. *et al.*, 1999) (Sealy-Lewis and Fairhurst, 1998)
malate synthase	**YES**	**Reported in S. cerevisiae** (Ordiz *et al.*, 1995)	*A. niger*	(Ruijter G.J. *et al.*, 1999) (Sealy-Lewis and Fairhurst, 1998)
Acetamidase	**YES**		*A. niger*	(Ruijter G.J. *et al.*, 1999)
Carnitine Acetyltransferase	**YES**		*A. nidulans*	(Stemple *et al.*, 1998)
phosphoenolpyruvate carboxykinase			*S. cerevisiae*	(Randez-Gil *et al.*, 1997) (Hynes *et al.*, 2002) showed it is not regulated by *facB* in *A. nidulans*
mitochondrial fumarate/succinate antiporter (*Arc1*)			*S. cerevisiae*	(Palmieri *et al.*, 1997)
Lactate transporter (*JEN1*)			*S. cerevisiae*	(Bogunga, 1999)

The induction by the acetate global regulator *(the *acuB* homologue *cat8)* of a dicarbocylic acid mitochondrial transporter, *Acr1* (mitochondrial fumarate/succinate antiporter), has only been identified and studied in *S. cerevisiae* (Palmieri *et al.*, 1997). Even so, its possible role in dicarboxylic organic acid transport across the mitochondrial membrane could be of great importance in organic acid production. This possible direct effect of *acuB* on organic acid production, as well as the wide and varied regulatory roles attributed to it in *A. niger* and closely related *A. nidulans*, made *acuB* an even more interesting gene deletion to study.

Mutants lacking *acuB* expression have been created in *A. niger*, but no instance of a full deletion has yet been reported. The mutants reported so far were identified as lacking *acuB* based on their lack of both isocitrate lyase and ACS activity, and their inability to grow on acetate ((Sealy-Lewis and Fairhurst, 1998); (Ruijter G.J. *et al.*, 1999)). The deletion strain used in this study was created using the new reversible auxotrophic parental strain background (see chapter 8). This allows for unparalleled precision in the study of the deletion, as the deleted strain is in all aspects identical to the parental strain (having no heterologous genes inserted in the genome and no auxothrophies), except for the intended replacement of the entire *acuB* gene with a 300bp scar.

9.2.   <u>Phenotype of the *acuB* deleted strain</u>

The created Δ*acuB* strain was tested alongside the WT on different carbon sources, in an effort to determine the importance of the *acuB* gene in the utilization of certain di- and tricarboxylic acids, as well as acetate. The difference in growth phenotype between the WT and Δ*acuB* strain was also tested on different concentrations of the toxic compound propionate. It has been reported that mutants in acetate metabolism are able to grow on concentrations of propionate at which the WT cannot grow (Sealy-Lewis and Fairhurst, 1998). Table 9.2 summarizes the results from these tests.

Table 9.2. Growth phenotype of the ΔacuB strain on different carbon sources compared to the WT. Tested by point inoculating spores of the WT and ΔacuB onto MM plates with the indicated carbon source.

Carbon source	Growth of ΔacuB	Growth of the WT
Glucose	**+++**	**+++**
Fumarate	**+++**	**+++**
Citrate	**+++**	**+++**
Succinate	**+++**	**+++**
Acetate	**---**	**+**
Propionate 4%, 0.1% glucose	**--**	**0**

+++   **Normal growth**

+   **Slower growth**

-   **Slow growth, altered morphology, delayed sporulation, Faun coloured spores**

--   **Very slow growth, altered morphology, delayed sporulation, Faun coloured spores**

---   **Very slow growth, altered morphology, no sporulation observed.**

0   **No growth**

It was interesting to observe how the Δ*acuB* strain was unaffected on citrate, fumarate and succinate as sole carbon source. This indicates that the role of *acuB* in organic acid production is more subtle, and more detailed physiological characterizations using batch fermentations were therefore conducted. Testing the Δ*acuB* strain on lactate as sole carbon source would however most likely have given an interesting result, as a Δ*cat8* (the homologue of *acuB* in *S. cerevisiae*) strain is unable to grow on lactate as sole carbon source.

9.3.   Deletion of *acuB* leads to physiological changes when *A. niger* is grown under glucose repressing conditions

The Δ*acuB* strain was compared to the WT and a control strain in batch fermentations in an effort to determine the possible effects of the deletion of *acuB*. The initial characterization was performed in Kristiansen (Kristiansen B. and Sinclair C.G., 1979) media, with a specific pH control profile (figure 9.1), a second characterization of the WT and Δ*acuB* was also performed in Pedersen (Pedersen H. *et al.*, 2000) media at pH6.
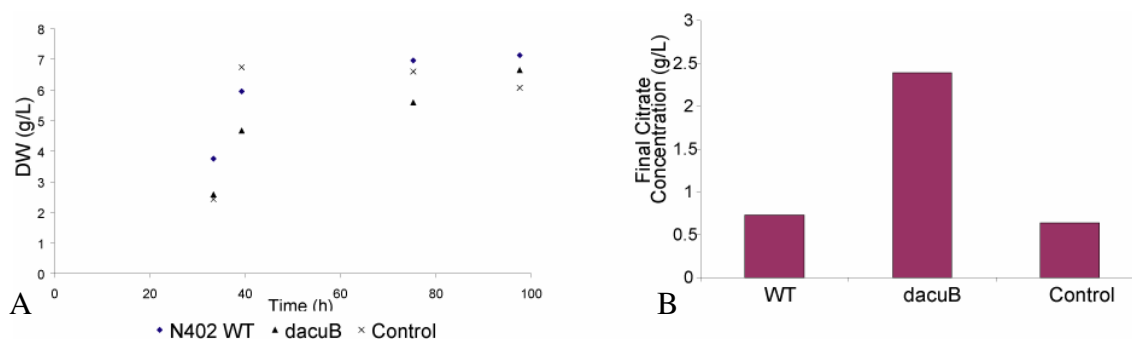
Figure 9.1. Kristiansen et al media (50g/L glucose), initial pH set to 2.5, after 18h the pH was increased to pH 5, and then allowed to decrease naturally. A: Dry weight profile for the WT, Δ*acuB* and the control strain (WT transformed with the pAN7-1 plasmid containing the *hph* marker). B: Final citrate concentrations achieved in the fermentations.

The dry weight profiles of the WT and control are similar, while the curve for the Δ*acuB* strain shows that it grew slightly slower (figure 9.1.A). The final biomass concentrations of the strains, however, are the same. It should also be noted that no acetate was detected in any of these strains. From figure 9.1B it can be seen that a higher final citrate concentration was achieved by the Δ*acuB* strain. The increased citrate production by the Δ*acuB* strain could be due to several factors related to the deletion of *acuB*. Possibly the availability of more intracellular acetate, the possible absence of certain futile cycles, or an unknown factor related to the role of *acuB* under glucose repressing conditions could be the cause. The changes observed in the level of citrate production when *acuB* is deleted also suggest system wide effects of this deletion. The increased level of citrate could also be due to decreased ACS or isocitrate lyase activity, which could lead to a

change in the mitochondrial ratio of citrate to isocitrate. The decreased demand for isocitrate could therefore favourably affect the accumulation of citrate and lead to higher citrate excretion from the mitochondria.

Ruijter *et al.* 1999 demonstrated the equimolar acetate/ oxalate yield of the *OAH* (oxaloacetate hydrolase) enzyme through partial purification of the enzyme. The lack of acetate production by *A. niger* during oxalate production was explained by showing that an *acuA* (encoding acetylCoA syntase) mutant did transiently produce acetate at pH 6. The ΔacuB strain was grown in Pedersen (Pedersen H. *et al.*, 2000) media at pH6 to test if the ΔacuB strain would produce acetate under similar conditions. It was found that ΔacuB did indeed produce acetate (at a maximum rate of 0.07 mM/gdw.h), while the WT did not produce any detectable amount of acetate under these conditions. The finding that an *acuA* mutant leads to acetate co-production with oxalate, also explains the production of acetate by the ΔacuB strain created in this study. From table 9.1, which describes the regulatory roles of *acuB,* it can be seen that *acuA* (encoding acetylCoa syntase) is induced by *acuB*. Lacking this induction, no acetyl-CoA syntase activity would be present, and acetate would therefore be produced through a similar mechanism as described by Ruijter (Ruijter G.J. *et al.*, 1999). Ruijter G.J. *et al.*, 1999 and Sealy-Lewis *et al.* 1998 demonstrated that ACS activity is not totally repressed by glucose, even though *creA* has been shown to repress it directly. It therefore appears that *acuB* is required for this basal level of ACS activity

In conclusion, the ΔacuB strain supported the results observed with acetate non-utilizing mutant strains (Ruijter, Seally-Lewis). The nature of the strain creation process also gives more significance to this data, and hopefully in future the ΔacuB strain (and the pyrimidine auxotrophic intermediate ΔacuB strain which was also created) can be used for more detailed analysis of the function of *acuB* under different conditions.

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Chapter 10. Conclusions

The aim of this work was to develop and apply the necessary metabolic engineering tools for the study, and eventual manipulation, of the central carbon metabolism towards enhanced organic acid production.

The initial aim of developing the necessary tools needed to efficiently apply metabolic engineering to *A. niger* was achieved through several projects:

1. Transfer of a cloning-free, PCR-based, bi-partite transformation method and a recyclable marker method from *A. nidulans* to *A. niger*, which allows for high transformation efficiencies and multiple rounds of genomic manipulation. These methods increase the rate of creation of mutant strains. Moreover, since the number of genomic manipulations are not limited by a limited number of transformation markers, it also allows strains of greater complexity to be developed. Such methods are essential if current advances, such as the sequencing of the *A. niger* genome, are to be used to full advantage.

2. Development of a reversible auxotrophic strain of *A. niger*, and proof-of-concept application of this strain in conjunction with the techniques transferred from *A. nidulans*. The reversible auxotrophic background strain enables the creation of strains with minimal disturbances to the genome. This allows for the study of a genomic manipulation in isolation, while preventing pleiotropic effects which might give misleading results. This method can also be used to improve the rate at which new strains containing multiple genomic manipulations are created in small- or large-scale strain improvement programs in an industrial setting.

3. Physiological study of *A. niger* using continuous fermentation and intracellular metabolite profiling. Continuous fermentation is a valuable tool for in-depth physiological studies of organisms and also specifically provides an excellent platform for investigating mutants with differing growth rates. Combining the high-level of control and reproducibility of continuous fermentations with intracellular metabolite profiling allows for detailed comparisons between mutants and wild type strains.

These tools were then applied to the problem of understanding and enhancing organic acid production in *A. niger*. Initially three strains with differing organic acid production profiles were compared using continuous fermentations and intracellular metabolite profiling. Clear differences between the strains (WT, glucose oxidase negative mutant strain and *Frds1* overexpressing strain) could be detected. Intracellular metabolite profiling revealed the effect of the enzymatic activity of *Frds1* in *A. niger* on the ratio between fumarate and succinate in the cell. Also, the extracellular metabolite profiles demonstrated the influence of *Frds1* insertion on citrate and oxalate production, respectively increasing citrate production and decreasing oxalate production under continuous fermentation conditions.

Further rounds of strain improvement were undertaken through the insertion of several genes involved in the reductive pathway of the TCA cycle (*MDH2, FumRs, Fum1s* and *Frds1*), either in combination or singularly. These strains were studied in batch fermentations. The main result from these insertions was the drastic increase of citrate production rates and yields achieved by the transformant strains.

The mechanisms of increased citrate production appear to centre on the mitochondrial malate/citrate antiporter. Increased cytosolic malate, caused by the insertion of *MDH2* or fumarase encoding genes, increased citrate production by increasing the cytosolic substrate for the mitochondrial citrate anti-porter. The increased citrate production linked to increased cytosolic succinate concentrations, both in batch and continuous fermentations with *Frds1* overexpressing strains, indicates that the mitochondrial citrate anti-porter can also utilize other cytosolic 4 carbon dicarboxylic acids, such as succinate, in addition to malate. These results also contribute to the mounting body of evidence on the importance of the mitochondrial citrate-malate anti-porter in citrate production.

O_2 limited fermentations were employed to study the effect of the inserted alternative cytosolic pathway (specifically the genes fumarase and fumarate reductase) on growth and metabolite production. It was found that, compared to the WT, the strains overexpressing both fumarase and fumarate reductase were better able to grow at very low oxygen conditions, and also produced half as much ethanol per gram biomass. The decreased ethanol yield indicates that *Frds1* plays a role in stabilizing the intracellular redox balance. Citrate production is dependent on the cell maintaining a redox balance while large quantities of NADH is produced through the TCA cycle, the redox stabilizing function of *Frds1* therefore appears to play a role in the increased citrate production observed for *Frds1* over-expressing strains.

Finally, the creation of the Δ*acuB* strain as part of the proof-of-principle application of the reversible auxotrophic system allowed this deletion strain to be compared to published information on *A. niger acu* mutants. Similarly to the mutant strains, the deletion strain was able to grow better than the WT on propionate, while growing very poorly on media containing acetate as sole carbon source. It was also found that the Δ*acuB* strain produced acetate during oxalate production in a comparable fashion to an *acuA* (ACS) mutant, and in contrast to the lack of acetate excretion by the WT. It has been shown that ACS activity is induced by *acuB* (Ruijters *et al.* 1999)*,* and the production of acetate by the Δ*acuB* strain therefore suggests that *acuB* is required for the basal level of ACS activity normally preventing acetate excretion by the WT strain.

Future prospects

It is my hope that the strains and methods developed as part of this Ph.D. will be further employed for the study of *A. niger*, as well as for further rounds of strain improvement towards more efficient organic acid producing strains. Specifically, using the reversible auxotrophy system to delete genes coding for enzymes leading to by-products such as oxalate and gluconic acid, as well as inserting the genes found by this study to enhance citrate production, could lead to strains with enhance production capabilities. The further elucidation of the citrate production pathway achieved during this Ph.D. should also allow for better strategies towards redirecting carbon flux in *A. niger*, especially with the added advantage of the released *A. niger* genomic sequence.

The current development of comprehensive genome scale stoichiometric *in-silico* models of *A. niger* should also in the near future reveal novel strategies towards redirecting carbon flux towards a desired product. However, it would most likely be necessary to do several genomic manipulations to achieve this, and the methods developed during this work should be well suited for such a task. The incorporation of other techniques, such as C-13 labeling and genomic array analysis of the created strains could also in future reveal more details of the under laying mechanisms, which led to the drastic increases in citrate production observed in this work.

Publications and presentations during this Ph.D.

Patent

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Papers

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De Jongh, W.A., J. Nielsen. (To be submitted upon approval of patent)**,** "Enhanced citrate production through gene-insertion in *A. niger*"

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Conference presentations

Oral presentations

Oral presentation at the conference: Physiology of Yeasts and Filamentous Fungi (**PYFF2**), Anglet, France, March 2004
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